

Regulation of Heat Shock Transcription Factor
in *Drosophila*

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To my brother Massoud

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Abstract

In response to heat shock and other cellular stressors, heat shock transcription factor (HSF) stimulates transcription of heat shock genes, a set of evolutionarily highly conserved gene family whose protein products are essential for many cellular processes including cell growth and development of thermotolerance. In higher eukaryotes, at onset of heat shock, HSF protein converts from a latent state, to a DNA binding one.

Both cytological and biochemical cell fractionation show that *Drosophila* HSF (dHSF) is cytosolic under normal condition and nuclear after heat shock. A heat-responsive nuclear localization sequence (NLS) in the dHSF was identified, with two interrelated functions suggested. The first is found in the basic residues which are essential for nuclear entry. Mutations in these residues completely block nuclear entry. The second function identified restrains the HSF in the cytosol. Two residues were found in the NLS which when altered allowed constitutive nuclear entry of dHSF independent of heat shock. These residues may interact with a putative cellular component or with other domains of the HSF to prevent nuclear entry.

The dHSF state of oligomerization, which is regulated by heat shock, was found to be dependent upon its subcellular localization. HSF when present in the cytosolic fraction of non shocked cells is monomeric and does not bind DNA. The nuclear HSF is trimeric and binds DNA even in absence of heat shock. The same mutations in the NLS that affected the subcellular

localization also affected the regulation of the oligomerization and DNA binding. The over expression of the dHSF resulted in its constitutive nuclear entry and DNA binding in the absence of heat shock suggesting the existence of a titratable inhibitory mechanism for HSF in the cytosol.

An activity which inhibited the DNA binding of the nuclear HSF *in vitro* was identified in the cytosolic fraction of cultured *Drosophila* cells and embryos. The inhibition is specific for the dHSF and is not due to a degradation of protein. This activity did not quantitatively transform trimeric HSF to the monomeric form *in vitro*. This and further *in vitro* analysis of the monomer-to-trimer transition of the dHSF suggest the existence of a multi-component complex for regulation of the dHSF's activity.

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Chapter 1

INTRODUCTION

Adaptation to suboptimal physiological and environmental conditions is an essential trait of living organisms. The ability to adjust to environmental changes not only allows organisms to survive a short period of unfavorable conditions, but it might also play an important role in the process of evolution if such changes are of sufficient duration. Mechanisms of cellular adaptation includes monitoring physical and chemical alterations in the environment and generating the proper responses. Cells of an entire organism constantly monitor changes in the surrounding environment through their specialized receptors that are localized either on the surface or inside the cell. The received information is processed through specific protein cascades that are organized in a sequential and coordinate fashion proceeding from the cell surface to the nucleus. Such cascades of proteins are linked together to form a chain of reactions; that is, they form a "signal transduction pathway."

A signal transduction pathway usually consists of a receptor, which receives and translates the incoming signal, and one or several signal transducers, proteins or small organic molecules that transfer the message to the site of response, the nucleus. A cellular response is often associated with a reprogramming of gene expression; that is, the expression of some genes are increased or induced while the expression of other genes are turned off or reduced. The protein products of newly induced genes make the cell undergo metabolic, physiological and structural changes, in order to adapt to the new

condition. In some cases this reprogramming causes cells to undergo cellular suicide (apoptosis) as a result of an intolerable condition.

The reprogramming of the pattern of gene expression is carried out by proteins referred to as "transcription factors." Transcription factors are proteins with a modular structure consisting of distinct and separable DNA-binding, oligomerization, nuclear localization signal, and transcriptional activation or repression domains (Mitchell and Tjian, 1989). The activity of any of these domains, together or separately, can be regulated by external signals (reviewed by Hill and Treisman, 1995).

Among the physical parameters of an environment, the temperature at which an organism, single cell or multi-cellular, lives is an essential feature of the growth condition. Variation in the temperature is sensed by individual cells of an organism and causes dramatic physiological changes both at the cellular and subcellular levels. The effect of elevated temperature in an organism at the molecular level was first reported by Ritossa (1962) where he observed a new puffing pattern on the polytene chromosomes of salivary gland cells as a result of a mild (24° C to 30° C) temperature shock. Further studies revealed that heat-induced puffs were sites of active transcription of specific genes later called heat shock genes (reviewed by Lindquist, 1986). Since then, the investigation of heat shock response has become a major area of research in biology and medicine. In this regard, the following questions have been the subject of numerous studies: i) how is a temperature shock

sensed by cells? ii) how is the heat-generated signal transduced to the cell nucleus? iii) which transcription factors are involved and how is their activity regulated by heat shock? iv) what is the nature and the mechanism of the cellular response?

Heat Shock Response

The term “heat shock response” is a historical reference to a general cellular response, the stress response. Stress response can be triggered by a diversity of chemicals and physiological conditions including heat shock, amino acid analogues, transition heavy metals, inhibitors of energy metabolism, ischemia, fever and inflammation, viral and bacterial infection, and aging (for a review see Morimoto *et al.*, 1992). Stress response is common to all organisms from bacteria to man (Lindquist, 1986).

Cells or organisms that are transiently exposed to 6-12° C above their normal growth temperatures undergo drastic changes both at the cellular and subcellular levels (Reviewed by Voellmy, 1994). At the cellular level, heat shock causes a total or partial transient arrest of the cellular proliferation and the associated processes (Thomas *et al.*, 1982; Welch and Suhan, 1986; for a review see Voellmy, 1994). One important cellular response is the development of thermotolerance; that is, after returning to the normal condition, cells develop an increased tolerance to subsequent heat shock or

other stresses. In other words, cells become capable of withstanding a level of stress that normally would be lethal (Welch and Suhan, 1986).

Associated with the changes of cellular processes is a dramatic reprogramming in the total pattern of gene expression. In general, RNA synthesis and processing as well as protein translation are affected by severe heat shock (Mayrand and pederson, 1982; Yost and Lindquist, 1986; Sierra and Zapata, 1994). While the expression of Heat Shock Proteins (HSPs) are induced both at the transcriptional and translational levels, the expression of a large number of proteins necessary for normal growth are suppressed (for a review see Lindquist and Craig, 1988). In the cytosol of the heat shocked cells, the pH declines rapidly (Weitzel and Rensing, 1985) and there is an increase in the level of calcium (Drummond *et al.*, 1986; Stevenson *et al.*, 1986). Another significant consequence is the decrease in ATP levels which may indicate a decrease in mitochondrial function (Findly *et al.*, 1983). The relationship among these sequence of events and their role in the cellular growth arrest and development of thermotolerance is not clear. Nonetheless it has been established that the induction of HSPs is necessary not only for survival of cells during the heat shock and other stresses but also for the development of thermotolerance (see Voellmy, 1994).

Heat Shock Proteins and Their Functions

Heat Shock Proteins (HSPs) are a family of proteins whose primary structures exhibit a remarkable degree of conservation among all organisms (Lindquist and Craig, 1988). HSPs were historically named according to their apparent molecular weight on denaturing SDS polyacrylamide gel (Lindquist, 1986). Major families of HSPs include the small HSPs ranging from 10 to 27 kDa, HSP60, HSP70, and HSP90 (Lindquist and Craig, 1988).

All HSPs, and a very close relative of them referred to as cognate heat shock proteins (HSCs), are present at low and moderate levels, respectively, in normally growing cells (Morimoto *et al.*, 1992; Voellmy, 1994). The synthesis of HSPs is induced, in a specific and coordinated fashion, up to a thousand fold in response to heat shock and other stressors (Morimoto *et al.*, 1992).

The high degree of sequence conservation of HSPs and the fact that the deletion of heat-inducible HSP70 in yeast is lethal suggest an important role for these proteins in cellular processes (Craig, 1990). Functionally, each HSP family has been linked to various cellular processes. For example, HSP70-related proteins are required for the development of thermotolerance and possibly play a critical role in the regulation of the cell cycle (Parsell and Lindquist, 1993; Rowley *et al.*, 1993). The over expression of HSP70 in *Drosophila* cells was observed to cause growth arrest at the G1 phase (Feder *et al.*, 1992). This correlates with the cell growth arrest at the G1 phase during heat shock and by prostaglandins which are known to induce the synthesis of

HSPs (Marui *et al.*, 1991). Mechanistically, in general, HSPs are thought to be protective to the cell by stabilizing macromolecular structures and reactivating inactivated enzymes (Skowrya *et al.*, 1990). Hsp70 and HSP60 function as molecular chaperones; that is, they assist other proteins to fold properly in an ATP-dependent fashion (reviewed by Hartl and Martin, 1992; and Hendrick and Hartl, 1993). HSP70 and HSP60 also assist in the translocation of proteins across the membrane of organelles within the cell (Deshaies *et al.*, 1988; Shi and Thomas, 1992). In addition, HSP70 is found in association with newly synthesized polypeptides suggesting a role in protein synthesis (Beckmann *et al.*, 1990).

Another HSP, HSP90, plays a pivotal role in several signal transduction pathways including steroid hormones, receptor tyrosine kinase and retroviral activation (reviewed by Rutherford and Zuker, 1994). HSP90 is found in association with steroid hormone receptors (Bohen and Yamamoto, 1994), the transforming tyrosine kinase pp60src (Brugge, 1986), and tyrosine kinase receptors Sevenless and Torso in *Drosophila* (Cutforth and Rubin, 1994). Association of HSP90 with these proteins is essential for their proper functioning (Rutherford and Zuker, 1994, and references therein).

Small HSPs, which are the least conserved HSPs (Lindquist, 1986) not only have a role in the establishment of thermotolerance, but they also are of clinical interest because of their role in drug resistance (Ciocca *et al.*, 1993). They have been found to be involved in embryogenesis of *Xenopus* and

Drosophila (Arrigo, 1987; Pauli *et al.*, 1990). One important feature of small HSPs is that they are induced not only by heat shock but also by steroid hormones suggesting a role for them in steroidogenesis (Thomas *et al.*, 1986; reviewed by Ciocca *et al.*, 1993). One of the small HSPs, HSP27, have been shown to be expressed in some estrogen-positive breast cancers (Edwards *et al.*, 1980) and its presence is correlated with the degree of tumor differentiation in endometrial carcinomas (Ciocca *et al.*, 1993).

Transcriptional Regulation of Heat Shock Proteins by Heat Shock Transcription Factor

The expression of HSPs in response to stress is induced mainly at the transcriptional level (Ashburner, 1970). Initial studies of the *Drosophila* HSP70 promoter revealed regulatory DNA sequences referred to as Heat Shock Elements (HSEs) which confer heat inducibility to this promoter (Pelham, 1982). Pelham and Bienz (1982) showed that when HSEs are fused to a heterologous promoter, they allow heat-induced transcription. HSEs consist of an array of repeats of the five base pair sequence nGAAn in which "n" symbolizes an A, C, T or G (Xiao *et al.*, 1991). Further studies showed that all heat shock genes contain multiple copies of HSEs in their promoter (reviewed by Lindquist, 1986).

The transcriptional activator that specifically binds to HSE was first identified in the nuclear extract of heat shocked *Drosophila* cells by Carl

Parker (Parker and Topol, 1984). The partially purified factor, referred to as Heat Shock Transcription Factor (HSTF, and in the recent literature HSF), was shown to be required for efficient *in vitro* transcription initiated from a *Drosophila* HSP70 gene template, as well as for protection of two HSEs upstream of the TATA box of this promoter (Topol *et al.*, 1985). Furthermore, it was shown that HSF binds to these HSEs in a cooperative fashion (Topol *et al.*, 1985; Xiao *et al.*, 1991). Subsequent to this, HSFs from other organisms such as yeast, human, mouse, chicken and tomato were isolated and molecularly cloned (Wiederrecht *et al.*, 1988; Sorger and Pelham, 1988; Clos *et al.*, 1990; Rabindran *et al.*, 1991; Sarge *et al.*, 1993; Schuetz *et al.*, 1991; Scharf *et al.*, 1990).

While yeast species- *S. cerevisiae*, *K. lactis*, *S. pombe* -and *Drosophila* each contain a single gene for HSF, the higher eukaryotes contain at least three independent genes. Two HSFs from human and mouse, three from chicken and tomato, have been thus far cloned (Schuetz *et al.*, 1991; Sarger *et al.*, 1991; Nakai and Morimoto, 1993; Scharf *et al.*, 1990). In species with multiple HSFs, only one of these factors (HSF1) has been shown to respond to heat shock (for a review see Voellmy, 1994). The exact function and regulation of those HSFs that do not respond to heat shock has not yet been determined.

HSFs from all organisms have in common that they all bind to the HSEs through the DNA-binding domain at their amino terminus, although the degree of amino acid homology of this region among all HSFs is only about

30%. A second region of homology which is required for oligomerization (Sorger and Nelson, 1989) is the region adjacent to the DNA-binding domain. This region consist of repeats of leucine and isoleucine residues (so called heptad repeats). Heptad repeats (also called leucine zipper) of other proteins--e.g., transcription factors--are shown to mediate homo or hetero multimerization through α -helical coiled-coil structures (reviewed by Vinson and Garcia, 1992). Other than the DNA-binding and oligomerization domains, the overall sequence comparison of all HSFs do not show a significant degree of homology. The HSFs from higher eukaryotes, however, contain a third region of homology at their carboxy terminal regions which is not shared with HSFs from *S. cerevisiae* and *K. lactis* (Clos *et al.*, 1990). This region consits of a hydrophobic leucine-isoleucine repeat (Clos *et al.*, 1990). Considering the conservation of the heat shock response and HSPs throughout evolution, one would have expected a higher degree of sequence homology among HSFs from different species. This low level of sequence conservation might reflect the notion that the mechanism of regulation of HSFs in different species have evolved in separate ways.

The mode of regulation of HSF in response to heat shock in different organisms has been the subject of several studies. In general, for a group of specific transcription factors that respond to extracellular signals, it has been shown that the activity can be modulated at several steps including cytoplasmic sequestration, DNA binding, and transcriptional activation or de-

repression (for a review see Hill and Treisman, 1994; see also introduction to chapters 2 and 3). Therefore, it is reasonable to expect similar regulatory steps for HSF. In the case of the HSF from *S. cerevisiae* (ScHSF), the first HSF that was cloned (Wiederrecht *et al.*, 1988; Sorger and Pelham, 1988), it has been shown that this protein is associated *in vivo* with promoter of HSPs in non-shocked cells (Jakobsen and Pelham, 1988). In addition, yeast extracts prepared from non-shocked and heat shocked cells exhibit similar HSE binding activities *in vitro* (Sorger *et al.*, 1988). These observations indicate that the DNA binding of ScHSF is not regulated by heat shock. Further research revealed that the transcriptional activity of ScHSF is controlled by heat shock (Nieto-Sotelo *et al.*, 1990; Sorger, 1990). Nieto-Sotelo *et al.* (1990) showed that the mechanism of heat-dependent activation of ScHSF includes the de-repression of a transcriptional activation domain of the protein. The molecular mechanism of this heat-dependent de-repression is not yet clear. HSFs from other organisms such as *Drosophila* and mammals show a different mode of regulation. *Drosophila* HSF and human and mouse HSF1 were found in a monomeric, inactive state in the whole-cell or cytosolic extract of cells growing in their normal growth condition (Zimarino and Wu, 1987; Clos *et al.*, 1990; Westwood *et al.*, 1991; Baler *et al.*, 1993; Sarge *et al.*, 1993). This form of HSF was incapable of binding HSEs *in vitro*. Heating such extracts *in vitro* resulted in the oligomerization and acquisition of the DNA binding state of HSF (Mosser *et al.*, 1990; Westwood *et al.*, 1991). These

experiments demonstrated that the oligomerization and DNA binding activity of HSF in insect and mammals are regulated by heat shock. These observations were further confirmed when the DNA binding activity and the oligomerization of HSF presence in the extracts of non-shocked and heat-shocked cells were compared (Baler *et al.*, 1993; Sarge *et al.*, 1993).

Because the inactive, monomeric form of HSF was always found in the cytosolic fraction of non-shocked cells, immuno cytochemistry method was used to examine whether a change of the subcellular localization of HSF is also a part of the regulatory pathway of HSF. In human and mouse cells, it was shown that HSFs 1 and 2 are primarily localized in the cytoplasmic compartment and heat shock resulted in the nuclear localization of these factors (Baler *et al.*, 1993; Sarge *et al.*, 1993; Sheldon and Kingston, 1993). It is important to mention that heat shock does not induce the DNA binding of HSF2 (Sheldon and Kingston, 1993). For *Drosophila* HSF, it was reported that this factor was localized in the nucleus under all conditions (Westwood *et al.*, 1991). This observation was contrary to the biochemical fractionation analysis which showed that *Drosophila* HSF was present in the cytosolic fraction of the non-shocked cell (Clos *et al.*, 1990; Westwood *et al.*, 1991).

Because the knowledge of detailed mechanisms of regulatory steps as well as the functional domains of HSF seemed essential for the understanding of the signal transduction pathway of stress, I analyzed the various aspects of the regulation of the HSF in *Drosophila* tissue cultures. In chapter 2, I present a

detailed *in situ* analysis of the subcellular localization of the dHSF in cells as a function of heat shock. The heat-dependent nuclear localization signal of the dHSF was determined and the mutational analysis of the NLS revealed amino acids that are responsible for the sequestration of the HSF in the cytoplasm as well as amino acids that are responsible for the nuclear targeting.

In the chapter 3, the relationships between the subcellular localization and the oligomeric state as well as the DNA-binding activity of the dHSF are analyzed. It is shown that there is a direct correlation between the residency of the factor in the cytosol and its state of oligomerization. The cytosolic HSF was found always to be monomeric. On the other hand, once the HSF became nuclear, be it as a result of heat shock or over expression in non-shocked cells, it was always oligomeric and bound HSEs with high affinity. Interestingly, mutations in the NLS region that decoupled the nuclear entry caused also the oligomerization and DNA binding of the HSF in the non shocked cells.

In chapter 4, it is shown that dHSF forms trimers as a result of heat shock. Furthermore, since conclusions of chapters 2 and 3 suggested the existence of an interaction between the HSF and a putative regulatory factor as a mechanism for the regulation of the cytosolic sequestration as well as the maintenance of the monomeric state, data are presented that support this idea. Attempts to identify and isolate the regulatory activity are also discussed.

In conclusion, this study shows that the primary level of the regulation of the dHSF's activity is the control of its subcellular localization. The next levels

of regulation include trimerization, DNA-binding, and activation of transcription. Thus similar to other transcription factors such as steroid hormone receptors and nuclear factor κ B, the regulation of dHSF's activity is achieved at multiple levels.

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Chapter 2

Identification and Mutational Analysis of a Stress Regulated Nuclear
Localization Sequence in the *Drosophila* Heat Shock Transcription
Factor

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Abstract

The subcellular localization of *Drosophila* heat shock transcription factor (dHSF) was analyzed in cultured cells by indirect immunofluorescence. We show here that dHSF is cytosolic under normal cell growth condition and becomes nuclear after heat shock. We identified a nuclear localization sequence in the dHSF that is responsible for the heat dependent nuclear entry. Mutational analysis of the NLS suggests that it possesses two interrelated functions. The first is in the basic residues which are essential for nuclear entry. Mutations in these residues completely block nuclear entry, as would be expected for a constitutive NLS. The second function identified is to restrain the HSF in the cytosol. Two residues were found in the NLS which when altered allowed constitutive nuclear entry of dHSF independent of stress. These residues may interact with a putative cellular component or with other domains of the HSF to prevent nuclear entry in normally growing cells. This NLS can function to target a β -galactosidase fusion protein into the nucleus in a heat-shock-dependent fashion.

Introduction

The regulation of nuclear transport is now known to play an important role in modulating the activity of certain specific transcription factors. Steroid receptors are localized in the cytoplasm in association with other proteins which might restrict their entry into the nucleus (Picard and Yamamoto, 1987; for review see Muller and Renkawitz, 1991; Hanover, 1992). When the appropriate hormone ligand is present, these complexes are disrupted and the receptor can enter the nucleus to activate transcription (Picard and Yamamoto, 1987; Muller and Renkawitz, 1991). Similarly, the NF- κ B/rel/dorsal family of transcription factors are tethered in the cytosol by association with I κ B proteins (Baeuerle and Baltimore, 1988; Beg *et al.*, 1992; Steward, 1989; for review see Baeuerle and Henkel, 1994; and Blank *et al.*, 1992). In response to the appropriate stimulus, I κ B becomes phosphorylated and degraded allowing NF- κ B to enter the nucleus (Beg and Baldwin, 1994; Henkel *et al.*, 1993).

In a similar way, human and mouse heat shock transcription factor 1 (HSF1) have been shown to respond to heat shock by oligomerizing and localizing within the nucleus (Baler *et al.*, 1993; Sarge *et al.*, 1993). It has also been demonstrated for human HSF2 that several hydrophobic potentially α -helical domains are important in regulating this process (Sheldon and Kingston, 1993). Cell fractionation studies in *Drosophila* are consistent with the multi-step activation process seen for the mammalian factors (Clos *et al.*, 1990; Zandi *et al.*,

1995). It has been previously reported that in non-shocked cells the *Drosophila* HSF (dHSF) is found in the cytosol as a monomeric protein which cannot bind DNA (Clos *et al.*, 1990; Westwood *et al.*, 1991). Upon heat shock the factor is found in the nuclear fraction in an oligomeric form which can bind DNA (Westwood *et al.*, 1991; Zandi *et al.*, 1995). Immunocytochemical analysis in the Westwood *et al.* (1991) report, however, showed the factor to be constitutively nuclear.

Regulation of nuclear localization is not a general phenomenon as most nuclear proteins enter the nucleus by virtue of the presence of a nuclear localization signal (NLS) present within the protein (for review see Garcia-Bustos *et al.*, 1991; Hanover, 1992). In general, nuclear proteins larger than 45 kD require an NLS for nuclear transport (Lanford *et al.*, 1986 and references therein). Two classes of NLSs have been identified (for review see Dingwall and Laskey, 1991; Nigg *et al.*, 1991). The well-characterized NLS of SV40 large T antigen consists of a proline residue followed by five basic residues with valine at the carboxy end (Kalderon *et al.*, 1984). The other class of NLSs are bipartite as was first shown for the *Xenopus* nucleoplasmin protein (Robbins *et al.*, 1991). This motif is comprised of two basic amino acids followed by a spacer region of any ten amino acids and a second basic cluster in which three of five amino acids are basic (Dingwall and Laskey, 1991). These bipartite NLSs have been found in a large number of nuclear proteins (Dingwall and Laskey, 1991).

In this report, and a related study (Zandi *et al.*, 1995), we show that the *Drosophila* HSF is regulated in a multi-step process that involves oligomerization and nuclear entry. We identify a stress-regulated NLS in the dHSF that possesses unique and interesting properties. Mutations in the NLS either block nuclear entry completely or allow constitutive nuclear entry. This NLS can function to target a β -galactosidase fusion protein into the nucleus in a heat-shock-dependent fashion. This domain of the dHSF also regulates the oligomeric state of the protein in the cytosol (Zandi *et al.*, 1995). When the NLS is deleted, the dHSF oligomerizes in the cytosol and can bind DNA (Zandi *et al.*, 1995). We present evidence for a titratable factor in the cytosol that interacts with this domain to restrain the factor in the cytosol in the absence of stress.

Results

Drosophila HSF Nuclear Entry is Regulated by Stress

The inactive monomeric form of HSF from vertebrates and *Drosophila* is present in the cytosolic fraction of non-shocked SL2 cells (Baler *et al.*, 1993; Sarge *et al.*, 1993; Westwood *et al.*, 1991; Clos *et al.*, 1990; Zandi *et al.*, 1995). Immunocytochemical studies of non-stressed HeLa cells and mouse 3T3 fibroblasts have shown that HSF1 is distributed in a diffuse pattern over the cytoplasm and nucleus (Sarge *et al.*, 1993). Upon heat shock, HSF1 is localized in the nucleus of both 3T3 and HeLa cells (Sarge *et al.*, 1993). In the case of the

dHSF, however, the immunocytochemical studies performed with *Drosophila* SL2 cells are inconsistent with the biochemical cell fractionation analysis of the non-shocked cells (Westwood *et al.*, 1991).

Our analysis of the localization reveals a pattern of dHSF localization that is very similar to that observed in mammalian cells. A combination of four monoclonal antibodies raised against the recombinant dHSF (Zandi *et al.*, 1995) were used to visualize the subcellular localization of the HSF in SL2 cells prior to and after heat shock. As shown in Figure 1A and B, in the non-shocked cells we found two kinds of staining patterns for dHSF. The pattern observed in Figure 1A shows an apparent perinuclear compartmentalization. In some cells this compartmentalization is clearly localized to one side of the nucleus, whereas in other cells staining is observed to be more evenly distributed surrounding the nucleus. The second kind of staining pattern observed is shown in Figure 1B. There is a rather diffuse staining pattern evenly distributed between the cytosol and the nucleus, similar to mammalian HSF1. In addition, strong staining can be seen over the region that corresponds to the nuclear envelope. The staining pattern observed in Figure 1B might be representative of cells that are partially stressed.

The occurrence of these two kinds of staining patterns depends on the experimental conditions. In the immunolocalization experiments shown in Figure 1A and 1B, cells were rapidly fixed on the tissue culture dish. This was done because we found that manipulating the cells prior to fixation as well as the

kind of fixative used often resulted in a partial or total nuclear localization of the HSF in the absence of heat shock. We also noted a significant variation in the kind of staining pattern depending upon the batch and the freshness of the formaldehyde solutions used. Very often, formaldehyde based fixatives would cause nuclear entry of the dHSF without heat shock (data not shown). We found, however, that a non-crosslinking fixative (Histochoice) gave significantly more reproducible results (see Materials and Methods).

After 20 minutes of heat shock, the HSF is no longer observed in the cytosol and nuclear envelope region; it is now almost exclusively localized within the nucleus in a punctate pattern (Figure 1C). The nature and composition of these granules are unknown. As a control for these experiments, we examined the intracellular localization of hsp83 in SL2 cells in response to heat shock. As shown in Figure 2A, hsp83 is cytoplasmic in cells grown at 25°C. When these SL2 cells are subjected to heat shock, hsp83 is distributed between the cytoplasm and nucleus (Figure 2B). These observations are comparable to those published by Carbajal *et al.* (1990), indicating that the Histochoice fixative does not cause artifactual compartmentalization.

The above analysis demonstrates that the dHSF is primarily localized in the cytoplasm in SL2 cells growing at normal temperature. These observations are in agreement with previous cell fractionation analysis of dHSF and with localization studies of mammalian HSFs. These results suggest that a stress-regulated nuclear localization domain may exist in the dHSF.

Identification of a stress-regulated NLS in the dHSF

To identify a potential stress-regulated nuclear localization signal within the dHSF, a series of carboxy as well as internally deleted dHSF mutants were constructed. Each of the mutant constructs were placed under the control of either the constitutive *Drosophila* actin 5c promoter or the CuSO₄ inducible *Drosophila* metallothionein (Mt) promoter (see Materials and Methods). Plasmids encoding these constructs were introduced into SL2 cells by calcium phosphate method (see Materials and Methods). To distinguish between the transfected and the endogenous HSF in these cells, an appropriate dilution of the anti-dHSF antibodies (α -dHSF) was determined, whereby only the exogenously expressed dHSF was visible (see Materials and Methods). When full length dHSF (construct N691) was expressed in normally growing SL2, cells its subcellular localization was dependent upon the expression level of the factor in cells (Zandi *et al.*, 1995). Generally, high expression levels generated by the actin 5c promoter resulted in constitutive nuclear localization of a significant fraction of the factor. This made it difficult to assess the role of potential nuclear localization signals (data not shown). Moderate expression levels were obtained by using the Mt promoter. Under these circumstances normal patterns of dHSF subcellular distribution were observed (Figure 3A). Upon heat shock, exogenous N691 is localized exclusively in the nucleus (Figure 3B) in a characteristic punctate pattern.

Identical conditions were then employed to determine the intracellular localization of ten HSF deletion mutants and a summary of the observed results is shown in Figure 3E. Progressive deletion of the dHSF carboxy terminus of amino acid 461 did not alter the subcellular distribution of the factor either in non-shocked or heat-shocked cells (Figure 3E constructs N586, N536, and N461). The subcellular distribution of two internally, and one amino terminal deleted HSFs, $\Delta 3h$, ΔBst and 33C, were also examined and the staining patterns observed were similar to those of N691 under normal and stress conditions (Figure 3E).

Our results also show that removal of the conserved leucine-isoleucine repeat (LIR) at the carboxy terminal region (construct $\Delta 3h$ in Figure 3E) does not affect the subcellular distribution of the factor in response to stress. Therefore, unlike the human HSF2, the LIR does not play an obvious role in regulating the subcellular distribution of dHSF. An internal deletion between amino acids 336 to 403 (Figure 3E, construct ΔSaf) and further carboxy deletion from 460 to 404 (Figure 3E, construct N404), however, resulted in the loss of the nuclear localization by the HSF under heat-shocked conditions). Further analysis of this region demonstrated that an NLS was localized between amino acids 393 to 420. Deletion of this region from dHSF (construct ΔNLS in Figure 3) completely decoupled the nuclear entry of dHSF in response to stress (Figure 3C and 3D).

It is interesting to note that none of the dHSF deletions examined so far allowed the constitutive nuclear entry of dHSF. It is possible that in addition to

functioning as an NLS, this region might contain a sequence which blocks nuclear entry in the absence of stress. Inspection of the primary amino acid sequence of the NLS suggests that it is similar to a bipartite NLS motif between residues 393 and 414. Deletion of residues adjacent to this segment from 414 to 424 resulted in a staining pattern similar to the full length dHSF (data not shown). Thus, we were unable to identify a regulatory function in the NLS by deletion analysis.

Nuclear Localization of β -Galactosidase-HSF Fusion Proteins

To further characterize the stress-dependent nuclear entry of dHSF, we constructed fusion proteins of *Escherichia coli* β -galactosidase with specific portions of dHSF. This system offers several advantages. First, β -galactosidase is a cytosolic protein and it does not localize to the nucleus by itself. Second, β -galactosidase (166 kD monomer) is well above the reported molecular weight (45 kD) of proteins that can freely traffic between the cytoplasm and nucleus (Lanford *et al.*, 1986). These fusion proteins thereby allow one to examine small portions of dHSF for their nuclear targeting ability.

The results of 11 such fusions are summarized in Figure 4E, and representative micrographs are shown in Figures 4A-D. Anti β -galactosidase antibodies were used for these immunostaining experiments. When β -galactosidase was expressed in SL2 cells, it localized in the cytosol under normal

or heat shocked conditions (data not shown). Fusions that include the entire HSF (Z-2C) shows a staining pattern very similar to full-length dHSF (N₆₉₁). As shown in Figure 4A, Z-2C is distributed evenly between the cytoplasm and the nucleus in non-shocked cells. Upon heat shock, Z-2C is detected exclusively in the nucleus (Figure 4B). The intracellular localization of fusions that include amino acids: 165 to 691 (Z-165C), 220 to 691 (Z-220C), 360 to 691 (Z-360C), 2 to 536 (Z-N536) and 2 to 460 (Z-N460) were all comparable to those of the Z-2C under both normal and heat shocked conditions (Figure 4E). In contrast, Z-Δ45 and all other fusions in which the region between residues 393 to 420 is deleted localized exclusively in the cytosol of both non- and heat-shocked cells (Figure 4C and D for Z-ΔNLS and 4E). These observations suggest that the region between residues 393 and 420 contains a stress regulated NLS. They further demonstrate that this element can function when fused to another protein and may therefore be independent of other dHSF sequences. It is important to note, however, that high levels of expression of these fusion proteins containing the NLS result in constitutive nuclear localization (data not shown).

Mutational Analysis of the NLS

The above deletion analysis suggests that the region between residues 393 and 414 is necessary and sufficient for stress-regulated nuclear entry of the dHSF. To characterize the NLS in more detail and to determine the residues involved in

stress regulation, this domain of the HSF was subjected to random mutagenesis analysis. A group of single and multiple amino acid substituted dHSFs were isolated as described in Materials and Methods. To properly assess the effects of the mutations on nuclear localization and stress regulation, stably transformed SL2 cell lines were established that carry each mutant HSF under control of the *Drosophila* Mt promoter. As shown elsewhere (Zandi *et al.*, 1995), we first determined the expression levels of dHSF where nuclear entry of the factor did not occur under non-shocked conditions. Based on these experiments, the Mt dependent protein expression in the cells was induced with 1 mM CuSO₄ for 1 hour.

Analysis of the subcellular localization of these mutant proteins revealed three classes of mutations. Class one mutations did not alter stress-dependent nuclear entry; however, the process of the nuclear localization was less sensitive to the experimental conditions, thus nuclear entry was induced primarily by heat stress. In addition, nuclear localization of this mutant could also be brought about by over expression (Zandi *et al.*, 1995). Class two mutations impaired dHSF nuclear entry under all conditions. Class three mutations affected the heat responsiveness of the NLS and allowed constitutive nuclear entry of the dHSF at normal growth temperatures. At the present time, there is only one example of the class one mutation. This mutant consists of three amino acid alterations; glutamine (Q) 399, lysine (K) 400, and leucine (L) 404 are changed to L, R, and R, respectively. The staining patterns of this NLS mutant are shown in Figure 5A

and 5B. In Figure 5A mutant L399R400R404 is localized exclusively in the cytoplasm of cells grown at 25°C. If the cells are heat shocked at 37°C, mutant L399R400R404 is localized within the nucleus in a diffuse pattern, as well as in the strongly staining punctate granules (Figure 5B). In class two mutants, the clusters of lysines (K) and arginines (R) in the NLS region (Figure 5O) are affected. In the cluster closest to the amino terminus, a double substitution of K400 to glutamic acid (E) and R401 to L resulted in the total impairment of nuclear entry (Figure 5I and 5J). Alteration of asparagine (N) 408 to serine (S) and R411 to L within the cluster closest to the carboxy terminus also blocked nuclear entry in either normal or stress conditions (Figure 5G and 5H). In addition, a single amino acid change from K405 to methionine (M) completely prevented nuclear entry (Figure 5C and 5D). One further single amino acid alteration of L404 to proline (P) (Figure 5E) of L404 to proline also prevented nuclear entry, suggesting the structural integrity of the NLS is also important for proper function (Figure 5E and F). All of the class two mutations caused the HSF to remain in the cytoplasm in a diffuse pattern regardless of growth temperatures. In addition, staining of the nuclear envelope which was observed for the wild-type factor was not observed when these mutations were present. This result suggests that interaction with a putative nuclear targeting protein which might concentrate the factor around the nuclear envelope was disrupted.

Class three mutants are particularly interesting in that they allow constitutive nuclear entry in the absence of stress. The residues affected include glutamine (Q) 403 and glutamic acid (E) 406. When Q403 or E406 are mutated to L and P, respectively, the mutant HSF enters the nucleus constitutively (Figure 5 K-L and 5M-N for P406). Constitutive nuclear entry of these HSF mutants suggests residues Q403 and E406 play an important role in retaining the HSF in the cytosol in the absence of stress.

Analysis of the β -Galactosidase-NLS Fusions

To further demonstrate that the region containing the NLS can function autonomously and independently of other HSF sequences, fusion proteins were constructed which join β -galactosidase and residues 393 to 424 of either the wild-type NLS or two singly mutated forms, K400 to M and Q403 to L. The subcellular distribution of these fusion proteins in SL2 cells was analyzed with representative micrographs and a summary of the results shown in Figure 6A-G. The fusion Z-NLS (wild-type NLS sequence) is primarily cytoplasmic under low level of protein expression in the non-shocked cells (Figure 6A). If Z-NLS was expressed in cells at high levels, a partial or total nuclear localization of the fusion was observed in the non-shocked cells. Z-NLS was, however, always localized within nuclei in heat shocked cells (Figure 6B). The fusion Z-NLS K405 to M was detected exclusively in the cytosol of either non-shocked or heat

shocked cells (Figure 6C and 6D), even at high levels of protein expression. The fusion Z-NLS Q403 to L showed stress-independent constitutive nuclear localization pattern. Although Z-NLS Q403 to L was largely localized in the nucleus in the non-shocked cells, there were some examples where the fusion protein was evenly distributed between the cytoplasm and nucleus (data not shown). Usually, however, the number of cells with exclusive nuclear staining was significantly greater than those evenly distributed throughout the cell. The overall staining patterns observed with these three fusion proteins indicate that the region between residues 393 and 424 contains sufficient sequence information to regulate stress-dependent nuclear translocation.

Predicted Secondary Structure of NLS

Secondary structural predictions (see Material and Methods) for entire dHSF suggests a high probability of an α -helical structure in the NLS region (data not shown). Assuming that the region 395 to 414 (Figure 5O) would form an α -helix, we superimposed the amino acids of 395 to 414 containing the NLS region onto the predicted helical structure which is shown in Figure 7A. It is interesting to see that those positively charged amino acids whose mutation resulted in the impairment of nuclear entry are positioned to one side of this putative helix. Interestingly, amino acids whose mutation resulted in constitutive nuclear entry are positioned on the opposite side of the basic residues. The putative helix has

the characteristics of an amphiphatic α -helix positively charged on one side and negative to neutral on the other. The two predicted different surfaces of this helix may provide specific sites of interaction for potential nuclear targeting proteins and regulatory factors.

For better visualization, we utilized a computer model (see Material and Methods) in which the amino acids 395 to 414 were fit in a calculated α -helix structure (Mayo *et al.*, 1990). Two views of this computer model is shown in Figure 7B and C. In these views, K and R residues are colored with red while Q residues are colored green and E residues are given a blue color. From the side view (Figure 7B), it is apparent that the basic amino acids (K and R), are juxtaposed on one side of the α -helix. Interestingly, the Q403 and E406 are positioned close together on the opposite side of K405.

Discussion

In this report we have identified a nuclear localization signal in the *Drosophila* HSF which is responsible for stress-regulated nuclear entry of the factor. Mutational analysis of the NLS suggests that it possesses two interrelated functions. The first is in the basic residues which are essential for nuclear entry. Mutations in these residues completely block nuclear entry, as would be

expected for a constitutive NLS. The second function identified is to restrain the HSF in the cytosol. Two residues were found in the NLS which when altered allowed constitutive nuclear entry of dHSF independent of stress. These residues may interact with a putative cellular component or with other domains of the HSF to prevent nuclear entry in normally growing cells.

Analysis of the putative secondary structure of the NLS suggests that this region can adopt an α -helical structure. Assuming that this is true, we examined the relative positions of the mutations which allowed constitutive nuclear entry and compared them to those which prevented nuclear entry. The interesting observation is that the mutations which prevent nuclear entry map to the opposite side of the helix from those which allow constitutive nuclear entry. This raises the possibility that these surfaces might interact with separate components, perhaps simultaneously.

Overproduction of the wild type dHSF results in a several-fold increase in cytoplasmic concentration and simultaneous nuclear entry in the absence of stress. The cytoplasmic form of wild type dHSF retains its inactive monomeric state even when overproduced (Zandi *et al.*, 1995). This observation suggests that there is a limit as to how much dHSF can be restrained within the cytoplasm even with an intact NLS. It further demonstrates that even though the cytoplasmic levels are significantly increased, an inactive state is maintained. Deletion of the NLS prevents nuclear entry even at high levels of NLS deleted factor; however, this factor now oligomerizes and binds to DNA in the cytosol

(Zandi *et al.*, 1995). DNA binding will occur even at low levels of overproduction of the factor, levels which would normally accumulate in the cytosol with wild type factor. Additionally, point mutations which prevent nuclear entry also accumulate in the cytosol, oligomerize and bind DNA. Taken together these results suggest that there is a titratable cytosolic factor which restrains the dHSF in the cytosol and holds it in a monomeric conformation. The domain of the dHSF which interacts with the restraining cytosolic factor (at least in part) is the NLS.

Based on these observations several models for how the NLS might function are evident. The first model would involve the binding of a cytosolic factor to the NLS in such a way that it precludes the interaction of the basic residues with the nuclear localization machinery. The cytosolic component could in principle be the HSF itself. This possibility is made less likely by our observations with the β -galactosidase fusion proteins. There we observed that small regions of the HSF containing the NLS would target β -galactosidase to the nucleus in a stress-dependent manner. This makes it unlikely that other regions of the HSF need to interact with the NLS to regulate its activity. Furthermore, the only region which, when deleted, affected the nuclear localization of the HSF was the NLS. Similarly, point mutations in the NLS which altered function in the intact HSF resulted in identical effects when fused to β -galactosidase.

These observations make it likely that a second component other than the HSF itself is responsible for regulating stress-dependent nuclear localization. This second component might consist of an unknown cytosolic retention factor. This retention factor might associate with the HSF through a specific surface of the NLS that would include residues Q403 and E406. By doing so it would not necessarily have to physically block or mask the basic residues on the opposite side of the NLS to prevent nuclear entry. It is, however, certainly possible that a masking phenomenon does occur to prevent nuclear entry in the absence of stress. The putative retention factor might itself directly respond to stress by altering its conformation or may be subjected to enzymatic modifications that are stress-induced. Alternatively, the NLS may itself be directly targeted by a stress-regulated enzymatic modification. We note, however, the absence of serine and threonine residues in the NLS.

Mutating L404 to a proline residue in the NLS prevents nuclear entry. This mutation would likely alter the secondary structure of NLS without changing the charge in the region. It is possible that the structure of the NLS is critical for nuclear targeting. With this in mind, the retention factor might itself hold the NLS in a conformation which does not permit the nuclear transport machinery to properly interact with the NLS. When stress occurs the retention factor may restore the conformation to a state that permits association with the nuclear transport apparatus. The mechanism for the switch could be an enzymatic alteration of the retention factor resulting in its release from the NLS.

The observations presented here and in a related report (Zandi *et al.*, 1995) are consistent with a cytoplasmic regulatory factor being involved in retaining the HSF in the cytosol. As described in Zandi *et al.* (1995), progressive over-production of the HSF in SL2 cells results in nuclear entry in the absence of stress. This result suggests the presence of a titratable cellular factor that is limiting in the cytosol which retains the HSF. Mutational analysis in the NLS also supports this idea.

Also demonstrated in Zandi *et al.* (1995) is the interesting observation that deleting the NLS results in the ability of the cytoplasmic factor to oligomerize and bind DNA. This is not a property of wild-type dHSF. Over-production of normal dHSF leads to a several-fold increase in cytoplasmic HSF, as well as constitutive nuclear entry. Only the nuclear form and not the cytoplasmic form can oligomerize and bind DNA. Clearly, the NLS region plays a key role in retaining the HSF in the cytosol in an inactive monomeric form. The next phase in the analysis of this level of HSF regulation will be to identify the cytoplasmic factor(s) which interacts with the NLS. Candidate proteins might include hsps or as yet unidentified proteins.

The studies presented here add further evidence for the existence of a multi-step regulatory pathway in the activation of the *Drosophila* HSF. Identification of the components of the activation process should help to further understand the mechanism regulating the stress signal transduction pathway.

Materials and Methods

Plasmids and DNA constructions

Two cDNAs for dHSF were isolated from a 0-20 hours embryonic cDNA library by PCR (CSP). These two cDNAs were identical except for their 5' region. One cDNA is truncated at the 5' end. This truncation removes 33 amino acids at the amino terminus of the HSF. The sequence of both cDNAs matches exactly the published sequence for dHSF (Clos *et al.*, 1990). Each of these cDNAs were subcloned as an *EcoRI* fragment into the pBluescript II SK (Stratagene) resulting in the pBSdHL (full length cDNA) and pBSdHs (5' truncated cDNA). The *Sall*-*PstI* fragment, containing HSF cDNA, of each pBSdHL and pBSdHs were subcloned into the *Sall*-*PstI* of the pSctgalx355GC (Rusconi *et al.*, 1990) resulting vectors pSctdHL and pSctdHs.

The *Drosophila* expression vector pPac (Krasnow *et al.*, 1989) was modified by inserting the double stranded form of the oligonucleotide (5'-GATCGGATATCTCTAGAGAGCTCTCCGGATGGGATCCAAGGCCTACGTATAAGTAACTAAC-3') in the *BamHI* site. This oligo contains multiple cloning sites and three stop codons at its 3'-end. This vector is called pPacO.

The CuSO₄ inducible expression vector (pMtdH) was made by replacing the *EcoRI* - *XbaI* promoter fragment of the pPacO with the *Drosophila* metallothionein (Mt) promoter fragment (Otto *et al.*, 1987), *EcoRI* - *XbaI*, from pHSH (a generous gift from Dr. Kevin Johnson) plasmid. The *XbaI* - *SacII*, which contains the 5'

coding region of the HSF cDNA in the pMtdH was replaced with the *XbaI* - *SacII* from pETHisdH to result the vector pMthisdH. The pETHisdH was made by subcloning of *NdeI* - *BamHI* fragment, which contains the full length cDNA of the dHSF, into the corresponding sites of 6His-pET 11d. In this construct the second amino acid of HSF is fused in frame to the six Histidines. This allows the isolation of the factor on the Ni-agarose (QIAGEN) column.

pPacOHyg was constructed as the following: oligonucleotides: 5'-CCCGGATCCAAAAAGCCTGAACTCACC-3' and 5'-CCCTCTAGACTATTCCTTTGCCCTCGG-3' were used to generate a PCR-fragment of the hygromycin B phosphotransferase gene from the pCophyg plasmid (a generous gift from Dr. Don Rio). This fragment was digested with *BamHI* and cloned into the *BamHI*-*StuI* of the pPacO.

Deletions were made at the carboxy terminus of HSF by cutting the pSctdHL at a *SphI* site, which truncates the protein at Glu-589, a *StuI* site, which truncates the protein at Gln-536, a *BspEI* site, which truncates the protein at Ser-461, a *AflII* site, which truncates the protein at Leu-404, or *SspI* site, which truncates the protein at Asn-338. The *SphI* site was blunt ended with Mung bean Nuclease and the *BspEI* and *AflII* sites were filled in using the large fragment of DNA polymerase. The internal deletion Δ Bst was made by inframe fusion of the *BspEI* and *StuI* sites in the HSF. Each of these plasmids was then digested with *XbaI* and appropriate fragment was gel purified and cloned into the *XbaI* - *SnaBI* of pPacO and pMt vectors.

The internal deletions $\Delta 3h$ ($\Delta 580-620$), ΔSaf ($\Delta 336-403$), and ΔNLS ($393-420$) were made using an oligonucleotide directed loop-out method by conditions very similar to the site-directed mutagenesis system of Kunkle *et al.* (1987). For each internal deletion, an oligo was synthesized in which the end points of the targeted sequences were brought together: $\Delta 3h$, 5'-GTCCATTAGATCAGAGTCTGAGGCCATCCTGAGGAG-3'; ΔNLS , 5'-AATATCTCCAGCATCACCATGGGCGTGGTA -3' ; ΔSaf , 5'-CTTGTTGTTCTCCTTAAG ACCGCCAGCGGTTCC-3'. Approximately 20 pmols of a phosphorylated nt was annealed to 0.3 μg of the single-stranded, uracil-substituted pBSdHL in 20 mM Tris (pH 8.0) 2 mM $MgCl_2$, and 50 mM NaCl. After annealing, 10x polymerization buffer (100 mM Tris-HCl at pH 8.0, 37.5 mM $MgCl_2$, 5 mM DTT, 7.5 mM ATP, 4 mM each dNTPs) was added to the reaction, as well as 10 units of T4 DNA ligase and 2 units of T4 DNA polymerase. The polymerization reaction was incubated for 15 min on ice followed by incubation 2 h at 37 C. The reaction was diluted to 80 μl with TE (10 mM Tris at pH 8.0, 2 mM EDTA), and 20 μl of that was used to transform *E. coli* strain DH5 α . The deletion of the targeted sequence was verified by restriction digest analysis. Using restriction sites at the flanking region of the deleted sequences, the homologous regions of the HSF in expression vectors pPacO and pMtHis were replaced. The joint points were verified by sequencing of the deleted region.

An oligonucleotide containing multiple inframe cloning sites (*Bam*HI , *Sma*I, *Afl*II, *Bsp*EI, *Xho*I) was inserted into the *Bam*HI -*Xho*I of pSctZ (a generous gift from Dr. S. Rusconi) resulting the vector pSctZE. The pPacoZE was constructed by ligation of *b*-galactosidase (*Z*) gene, *Xba*I-*Xho*I fragment from pSctZE into the pPaco. HSF- β -Galactosidase fusions were constructed as follow: The HSF deletions were generated by PCR and fused to the *Z* in the pPacoZE vector. All 5' primers contain a *Bam*HI inframe site and all 3' primers contain an inframe stop codon followed by a *Xma*I site. PCR fragments were digested with *Bam*HI and *Xma*I and cloned into the *Bam*HI and *Bsp*EI of pPacoZE. Primers B1 (5'-CCCGGATCCTCCAGGTCGCGTTCA-3') and X1 (5'-TTTCCCGGGTTACAACCTCGTG-3') were used to generate the entire dHSF coding region from the amino acid 2 to 691 including the stop codon. This fragment is called hereafter 2C. The resulting construct is called Z-2C in figure 4E. For Z-165C, in which dHSF from amino acid 165 to 691 is fused to *Z*, primer B2 (5'-CCCGGATCCGCCATGTCTGAAG-3') and X1 were used. For Z-220C primer 3B (5'-CCCGGATCCCAGTTCCTCATT-3') and X1 were used to generate dHSF coding region from 220 to 691. For Z-360C primer B4 (CCCGGATCCGATGTCTACACA-3') and X1 were used. For Z-405C, the 2C fragment was digested with *Afl*II and *Xma*I and ligated to the *Afl*II and *Bsp*EI of the pPacoZE. For Z-460C, the 2C fragment was digested with *Bsp*EI and *Xma*I which was ligated into the *Bsp*EI of the pPacoZE. The correct orientation of this fragment was determined by restriction digest analysis. For Z-N405 in which *Z* is

oligonucleotides having mutations at the second and third position of the codon for each amino acid. The w10 oligo was used for dsDNA synthesis by the method of Kunkel *et al.* (1987) as described above. After transformation of dsDNA into bacteria, single colonies were picked and plasmid DNA was isolated and the mutagenized region was sequenced. After identification of mutations, the mutated regions were subcloned (via *BstBI* and *BspEI*) into the pPacodH or pMtHisdH to replace the homologous portion of the dHSF with the mutated region.

Cell culture, stable and transient transfections

The *D. melanogaster* Schneider 2 cell line (SL2) (Schneider, 1972) were maintained in SL2 cell culture media (GIBCO) supplemented with 10% heat treated (at 65° C for 30 min) fetal bovine serum (Hy-clone) and 100U/ml penicillin and 100U/ml streptomycin. Cells were grown in Corning flasks at 25°C (non-shocked). For DNA transfection, 3×10^6 cells were seeded in 3 ml of medium on a 30 mm tissue culture dish and transfected using calcium phosphate precipitates (Ausubel *et al.*, 1992) of cocktail of 2 plasmides at indicated amounts along with 10 µg of pBluescript plasmid as carrier DNA. The cotransfection cocktail contained a mixture of 5 µg of the vector that contained the gene of interest and 1 µg of the vector that contained the selectable marker pPacHyg. At 15-18 hr post-transfection cells were spun down, washed once with 1x PBS containing 2 mM EDTA, and once with the complete medium, and resuspended

in 3 ml of fresh medium. After 4 days, cells were spun down and resuspend in selective medium containing hygromycin B (200 $\mu\text{g}/\text{ml}$). Selective medium was replaced every 5 days, and stably transformed polyclonal cell populations were isolated after 3 or 6 weeks of selection with hygromycin B. Hygromycin B was maintained routinely in the media at all times after selection.

For transient expression, cells were transfected as above, except no vector containing a selective marker gene was included in the transfection mixture and the amount of the DNA that was used for transfection is indicated in the figures. After transfection, cells were washed as above and grown for 2 days before using them for experiments.

Cells were heat shocked at 37° C for 30 min on the tissue culture dish by transferring the plates to a 37° C incubator.

CuSO₄ induction

We have analyzed and determined the optimum induction procedure for the Mt promoter. Cells were induced routinely by addition of 1 mM of CuSO₄ to the culture medium for indicated times in figure legends.

Cell staining and immunofluorescence detection

Cells were grown at 25° C and subjected to heat shock as described above. Non-shocked and heat-shocked cells were fixed directly on the tissue culture dish with the 1x tissue fixative "Histochoice" (Amresco, Solon, Ohio) for 15

minutes at room temperature (RT). Fixative was removed and cells were washed three times with 1x PBS (137 mM NaCl, 5 mM KCl, 7.5 mM Na₂HPO₄). Cells were incubated 1 minute in 0.1% NP40 in PBS. After 3 PBS washes, cells were incubated with a 1:100 dilution, for detection of the endogenous HSF, or 1:1000 dilution, for detection of the expressed factors, of a mixture of four monoclonal anti-*Drosophila* HSF antibodies or IgG fraction of rabbit anti beta-galactosidase (Capple, Oregon Teknika Corp., West Chester, PA), in 1% nonfat dry milk for 1 hour at RT. After extensive washing with PBS, cells were incubated with secondary antibodies, fluorescein-conjugated goat affinity purified anti mouse IgG-IgM, or rhodamine-conjugated IgG fraction goat anti rabbit IgG (Capple), at a dilution of 1:200 for 30 minutes at RT. Cells were washed four times with PBS and a drop of buffered 90% glycerol containing 2% propyl gallate was applied and a NO. 1 cover slip was attached. Cells were viewed and photographed using a Zeiss Axioplan under normal phase microscopy and with UV irradiation with the aid of a fluorescein and rhodamine specific filters. Micrographs were reproduced from slides on a sprint scan 35 (Polaroid) and printed on a Kodak XLS 8300 digital printer.

Theoretical structure analysis and computer modeling

The secondary structure prediction of HSF was done by using the Wisconsin package software. The computer modeling was done with the aid of the Biograf (Molecular Simulations Inc., Burlington, MA) program. In this program an

optimal α -helix is calculated with phi and psi angles set at -57° and -47° respectively. The hydrogen bonds between the turns of helix are set to the optimal 2.8 angstrom distance. Side chains in this helix are set to fully extended conformations. The amino acids 395 to 414 of the dHSF were fit in this program and viewed.

Acknowledgments

We thank Bassil Dahiyat for help in the computer modeling studies. We thank Gary Belford for his aid in reproducing the micrographs from slides.

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Figure legends

Figure 1. Localization of the *Drosophila* HSF in SL2 cells. The figure shows the immunofluorescent photomicrograph positioned on top of the phase contrast photomicrograph. (A and B) non shocked cells, and (C) cells heat shocked for 30 minutes at 37°C. In each panel two sets of photomicrographs are shown representing separate experiments. In this experiment cells were stained with a 1:100 anti-dHSF antibodies.

Figure 2. Localization of the *Drosophila* hsp83. The *Drosophila* hsp83 were visualized using monoclonal anti-hsp83 antibodies (Carabajal *et al.*, 1990) in SL2 cells growing at 25°C (A) and heat shocked at 37°C (B) by indirect immunofluorescence.

Figure 3. Subcellular distribution of dHSF deletion mutants. Immunofluorescence micrographs of non-shocked (25°C) and for 30 minutes at 37°C heat shocked SL2 cells in which recombinant full length dHSF (N691), A and B, and NLS deleted dHSF (Δ NLS), C and D, are shown. (E) The summarized results of immunofluorescence analysis of expressed dHSF deletions in SL2 cells. dHSF derivatives are schematically represented by stippled boxes with numbers indicating amino acid positions. The cyt represents staining patterns such as C or D indicating an exclusively cytoplasmic localization. The nu represents staining

pattern such as B indicating exclusively nuclear localization. The cyt/nu represents staining pattern such as A indicating an evenly distributed staining pattern between cytoplasm and nucleus. For each construct, cells were transiently transfected with plasmid encoding the appropriate recombinant factor under control of the Mt promoter. Expression of recombinant proteins were induced with 1 mM CuSO₄ for 1 hour. Recombinant factors were stained with a 1:1000 dilution of the anti-dHSF antibodies.

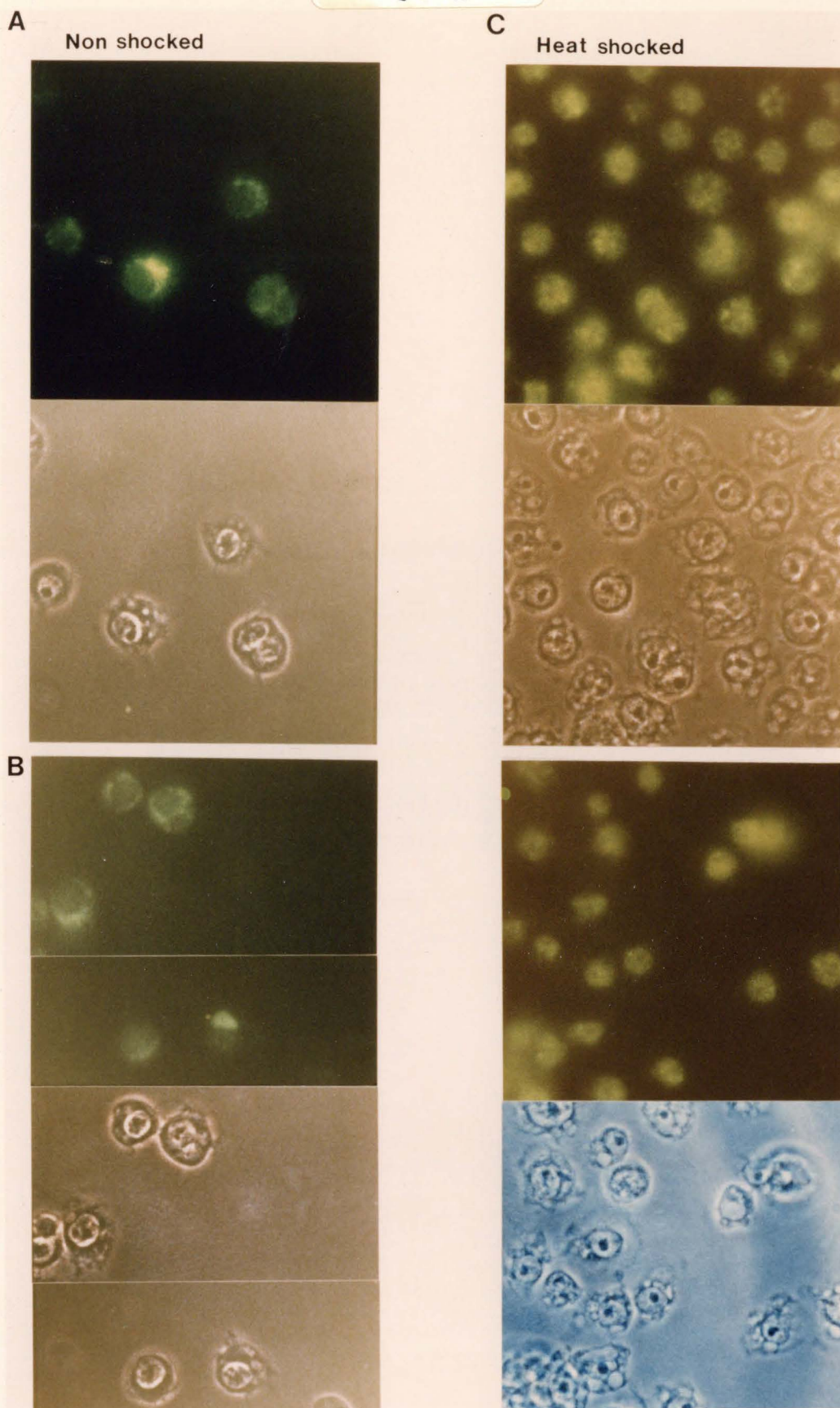
Figure 4. Subcellular distribution of β -galactosidase-dHSF fusion derivatives. Fusion proteins were expressed in SL2 cells by transfecting cells with 0.25 μ g of plasmid, encoding each of them under control of the actin 5C promoter. Fusion proteins were visualized in the non-shocked (25°C) and for 30 minutes at 37°C heat shocked cells using anti β -galactosidase antibodies as described in Materials and Methods. (A-D) Immunofluorescence of representative fusions Z-2C and Z- Δ NLS. (E) Summary of immunofluorescence analysis. The dHSF portions and β -galactosidase are represented by stippled and open boxes, respectively. Numbers above stippled boxes indicate N- and C-terminal amino acid positions of dHSF. For description of cyt, nu, and cyt/ nu, see legend of figure 3.

Figure 5. Mutational analysis of NLS. (A-N) Single or multiple point mutations were generated as described in Materials and Methods. Expression of

each mutant was induced with 1 mM CuSO₄ for one hour in stable cell lines carrying the appropriate constructs under control of Mt promoter. Non-shock (25°C) and at 37°C for 30 minutes heat-shocked cells were stained with anti dHSF antibodies as described in Materials and Methods. Nomenclatures of mutants: The first letter indicates the wild type amino acid, the number shows the position and the third letter is the mutant amino acid. Amino acids are in single letter code. (O) Functional domains of *Drosophila* HSF. The 691 amino acids of dHSF are represented by stippled box. Solid bars above it indicate functional domains that have been mapped previously (Clos *et al.*, 1990, Zandi *et al.*, 1995) and in the present work. The nuclear localization sequence is shown below the box.

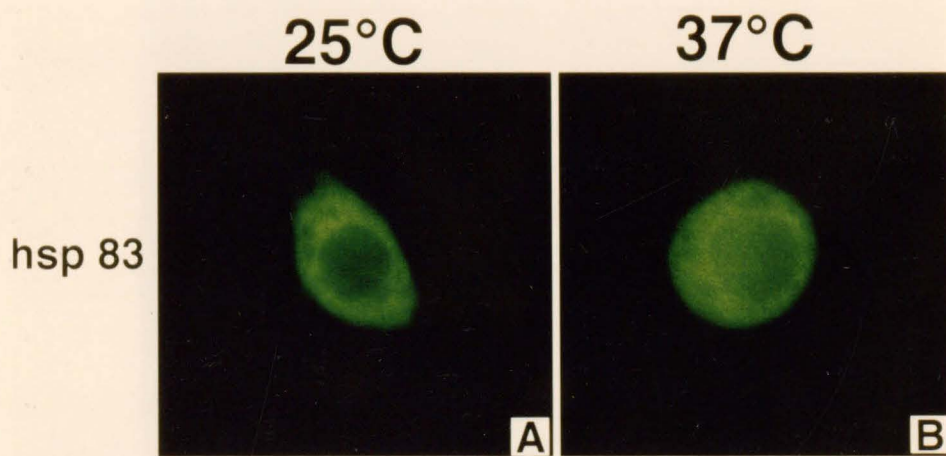
Figure 6. Analysis of β -galactosidase-NLS fusions. (A-F) Immunofluorescence micrographs of wild type NLS (Z-NLS), K405M-NLS (Z-NLSK405M), and Q403L-NLS (Z-NLSQ403L) fusions to β -galactosidase are presented. Conditions for cell transfection and staining are the same as described in the legend of figure 4. (G) Summarized results of A-F. Open box represents β -galactosidase and stippled box represents the NLS portion of dHSF. Cluster of basic amino acids are underlined. Amino acids which mutation resulted in the uncoupling of nuclear entry are indicated with a “*” on the top. Amino acids which mutation caused constitutive nuclear entry have a “bullet” on top.

Figure 7. Secondary structure analysis. (A) Superimposition of a helical wheel with NLS amino acids positioned on it. Amino acids which mutation disrupted nuclear entry are put in round boxes and amino acids which mutation resulted in the constitutive nuclear entry are put in square boxes. (B and C) Side (B) and top (C) views of computer modeling of the NLS. Red color represents basic amino acids (K and R). Green and blue colors represent glutamine and glutamic acid residues, respectively. In these views only lysine, arginine, glutamins, and glutamic acid residues are shown.



58

Fig. 2



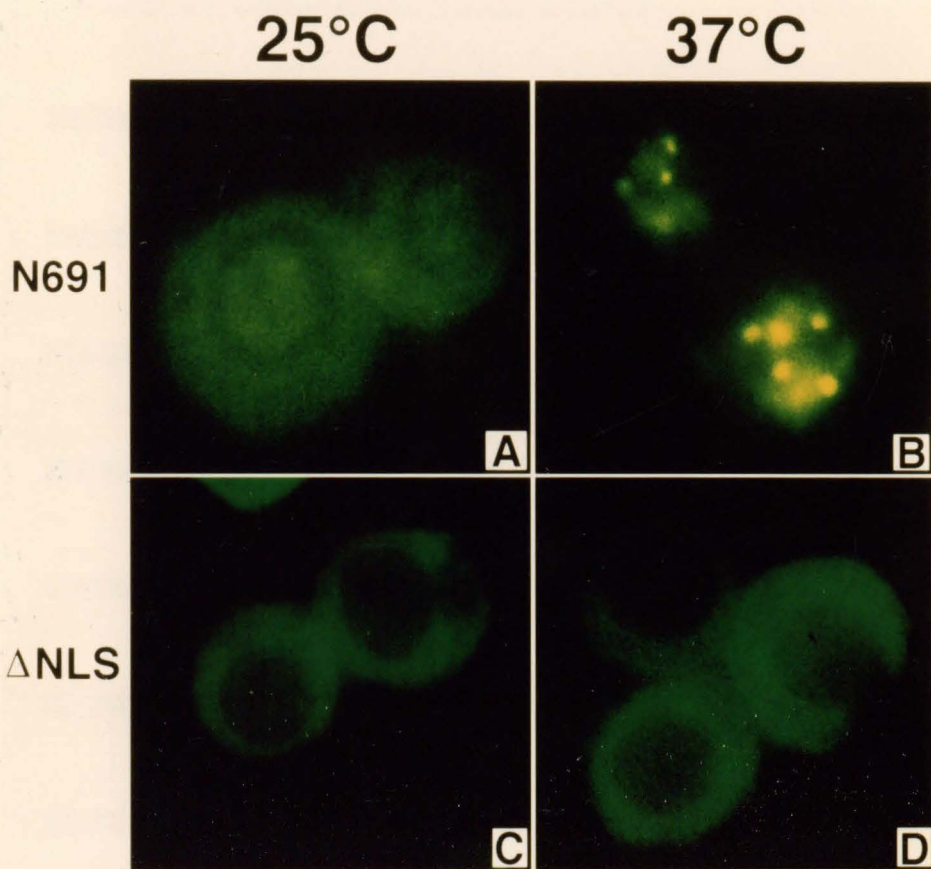
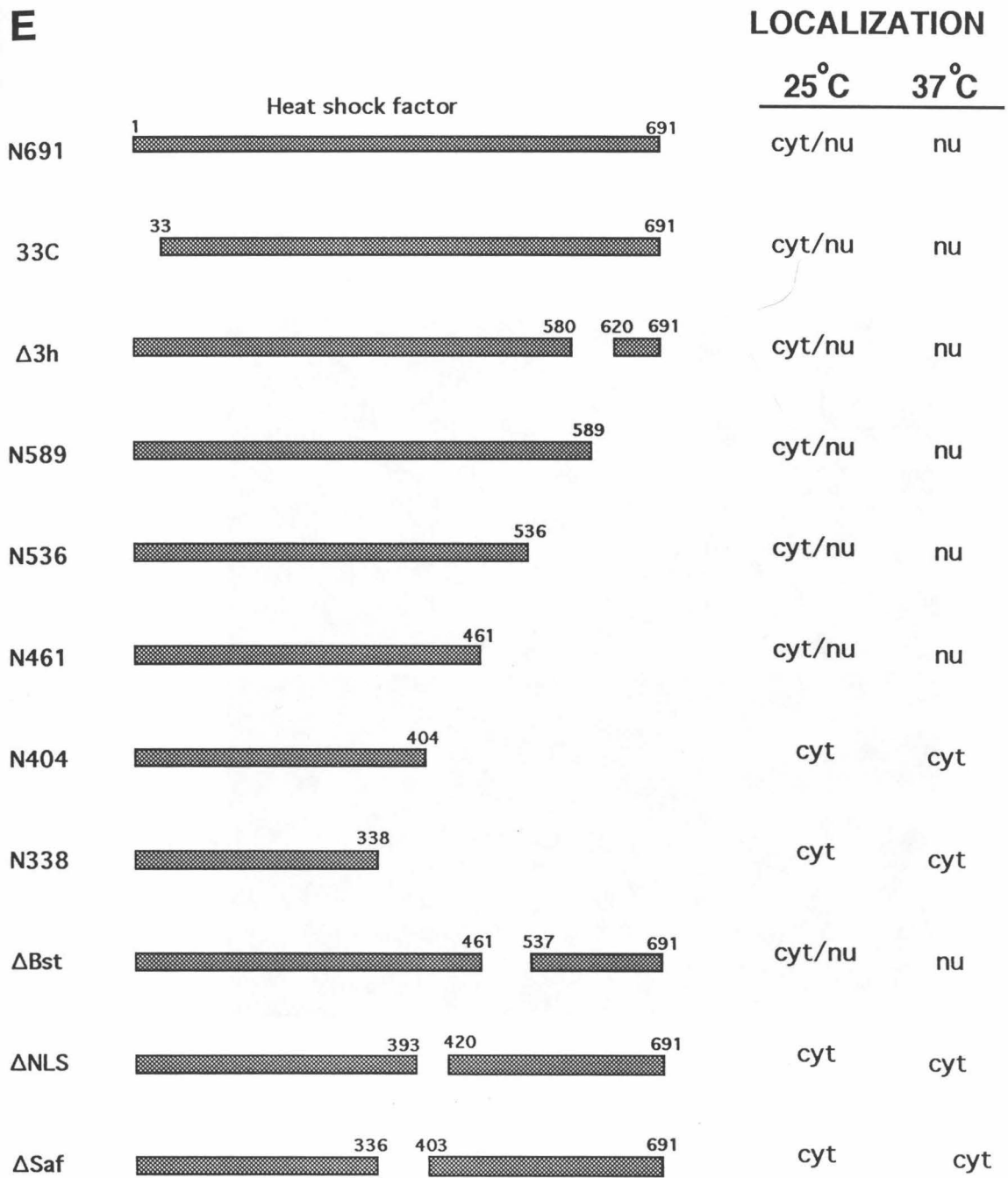


Fig. 3E



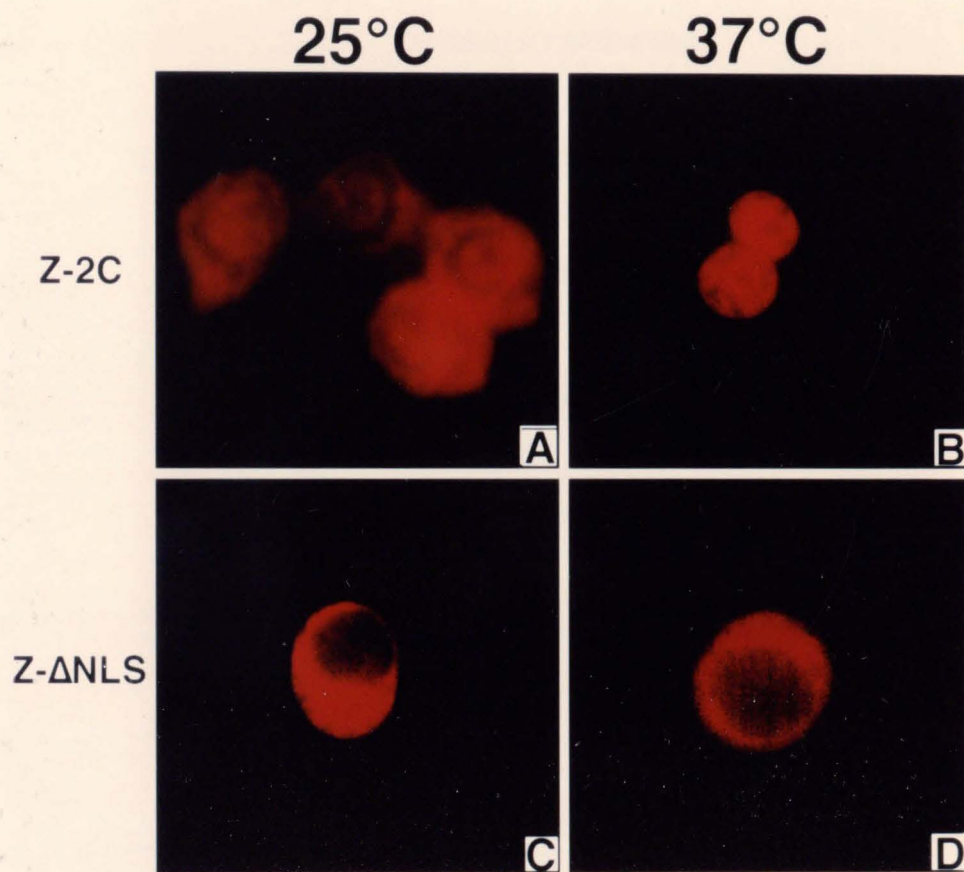
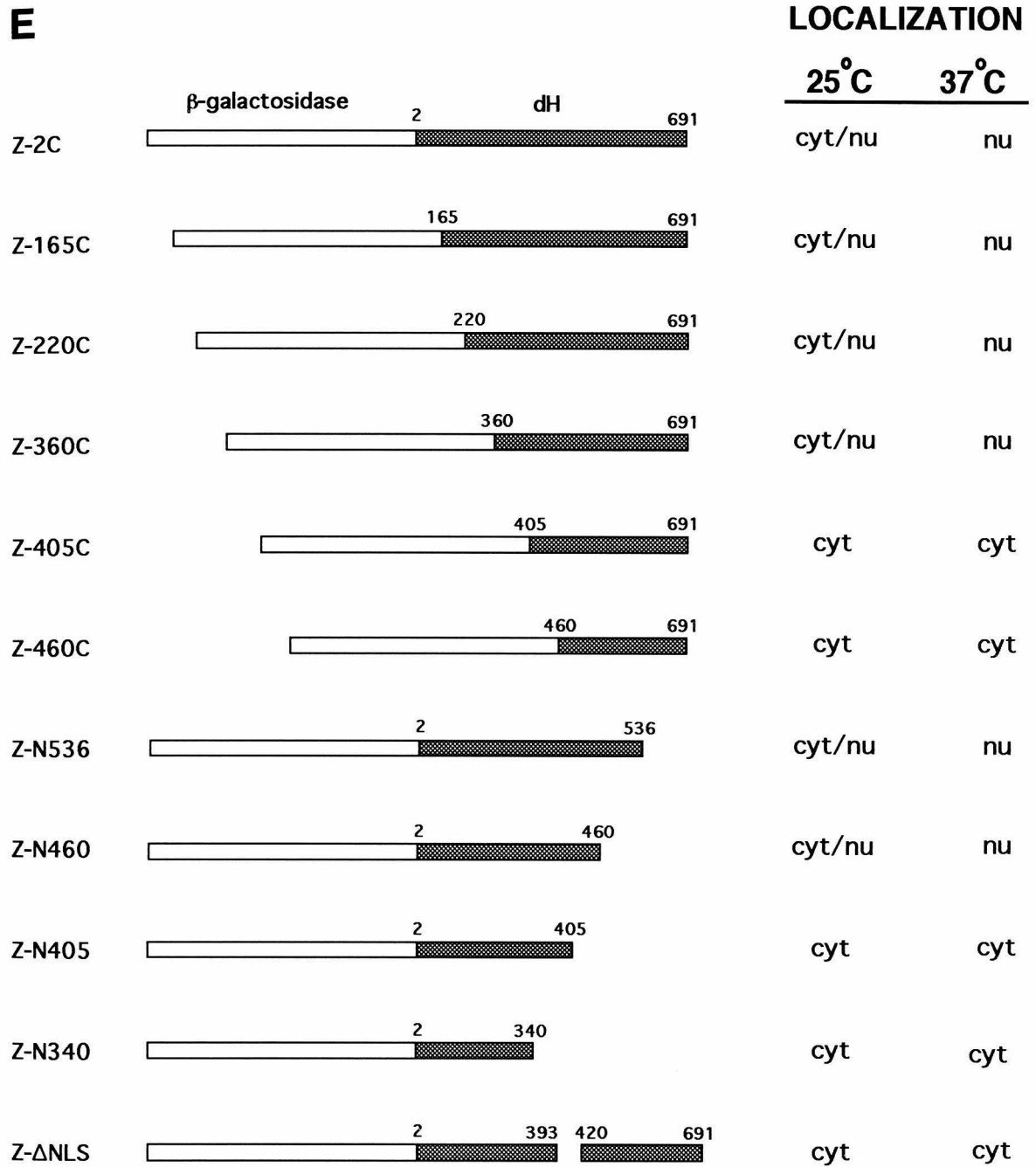
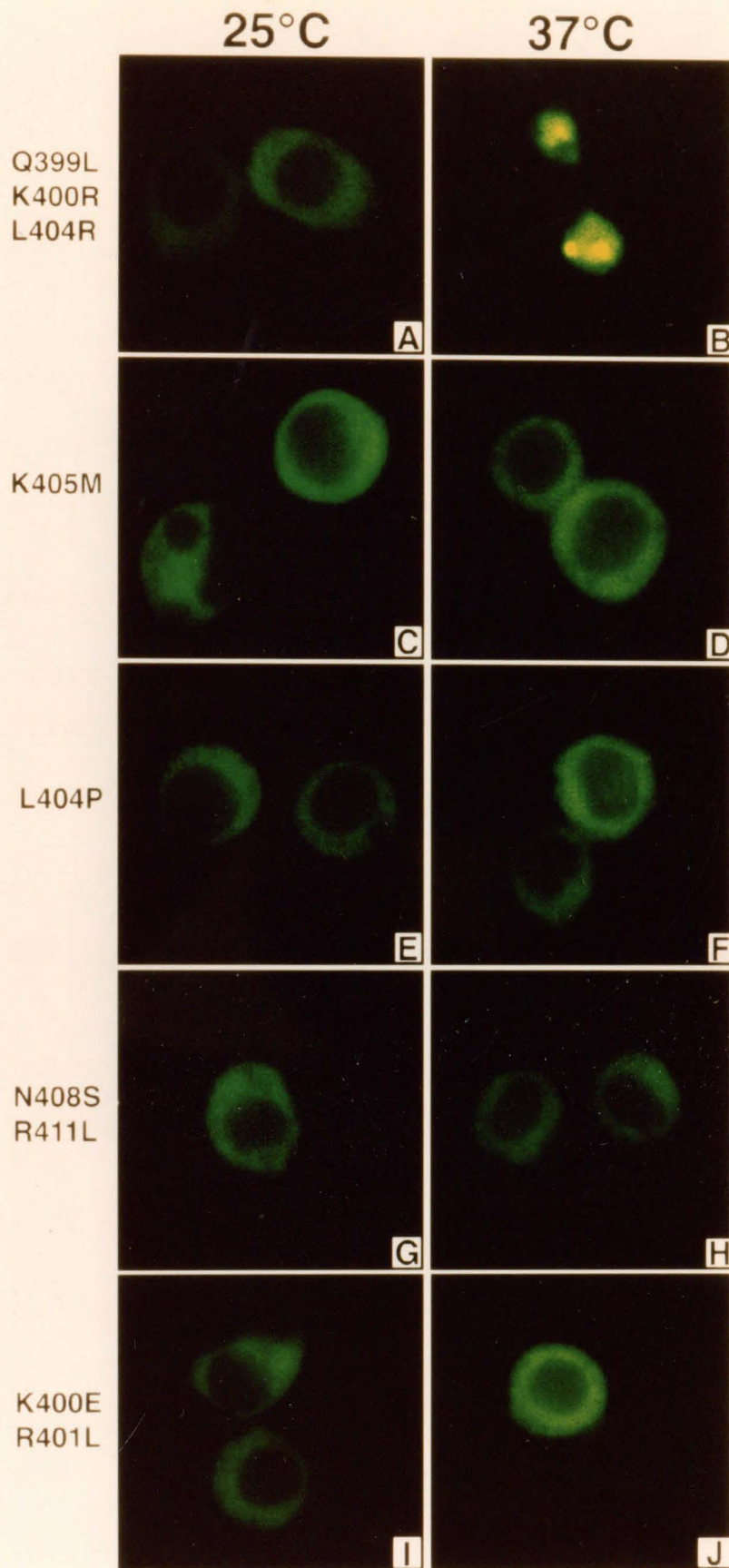


Fig. 4E





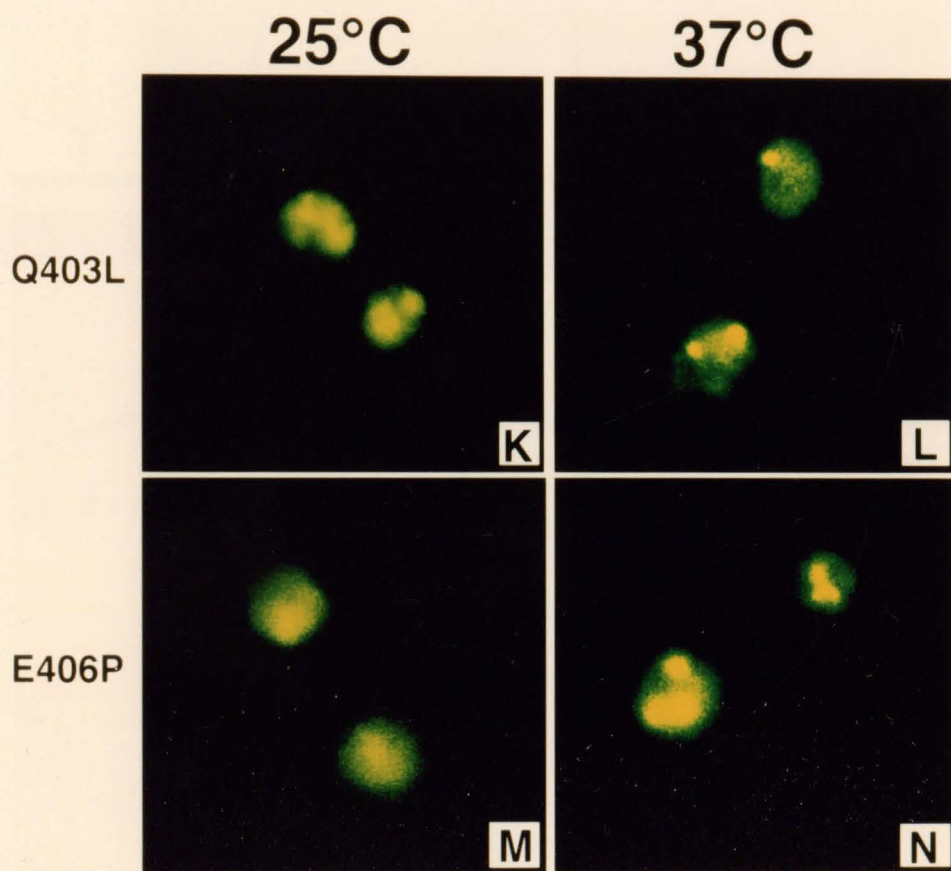
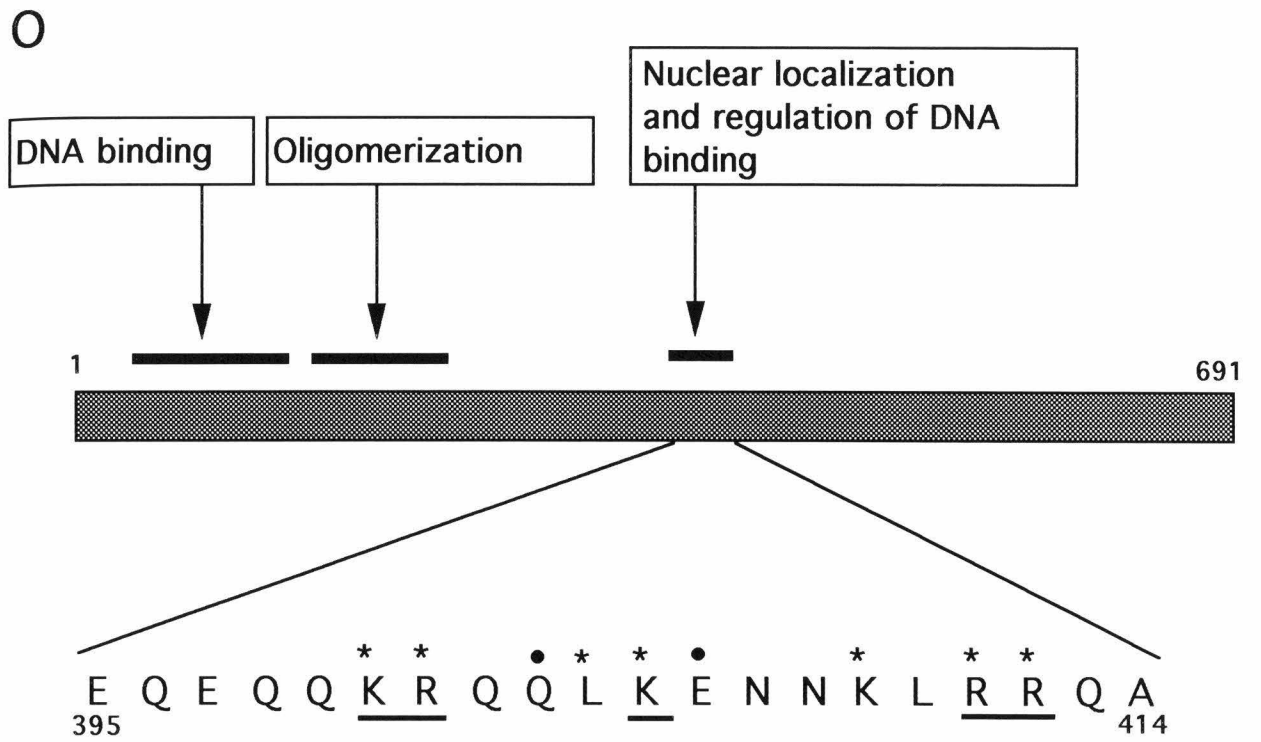


Fig. 5-O



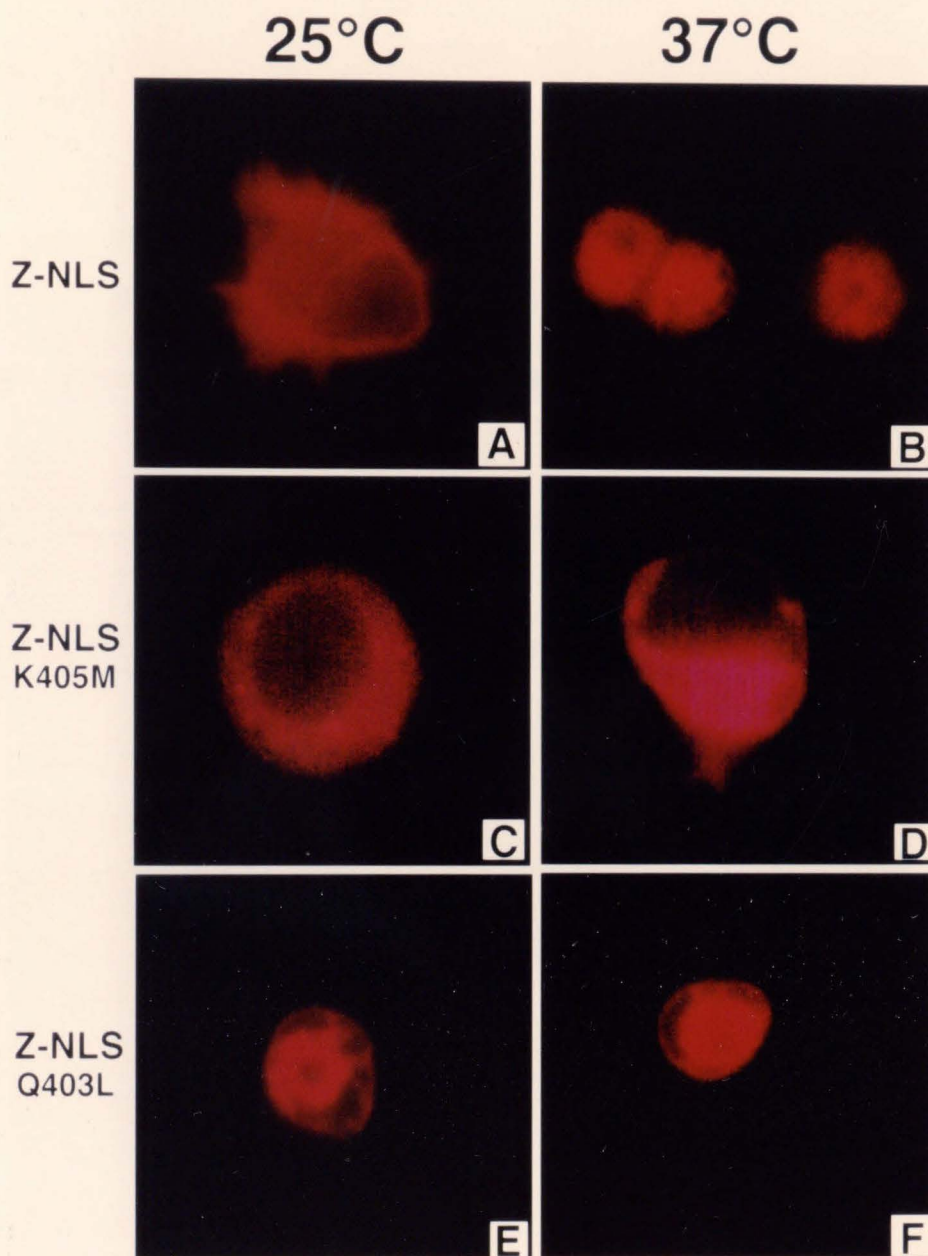


Fig. 6G

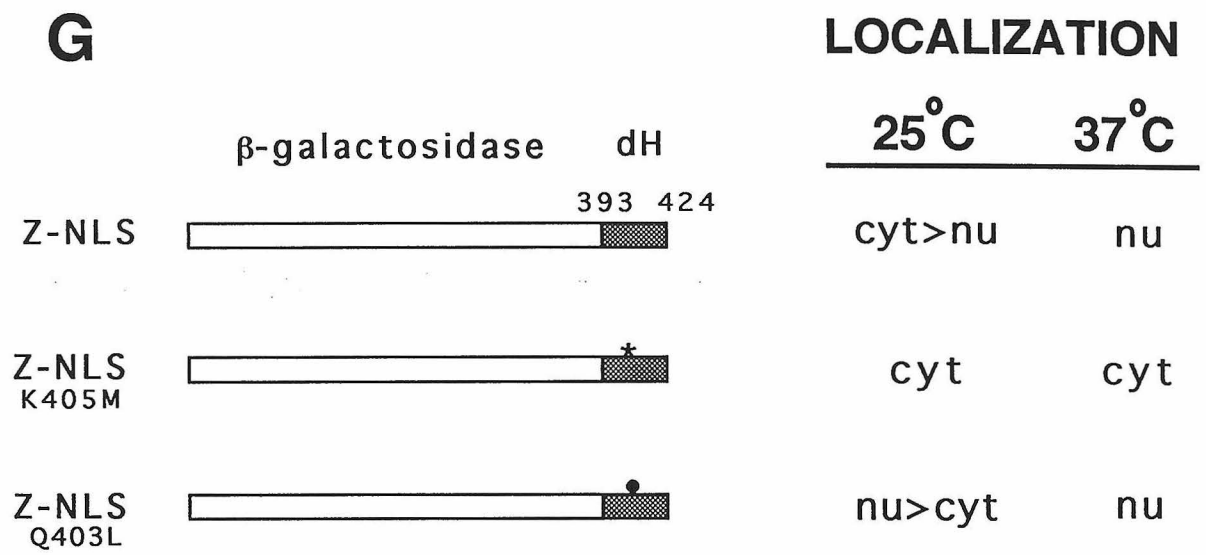
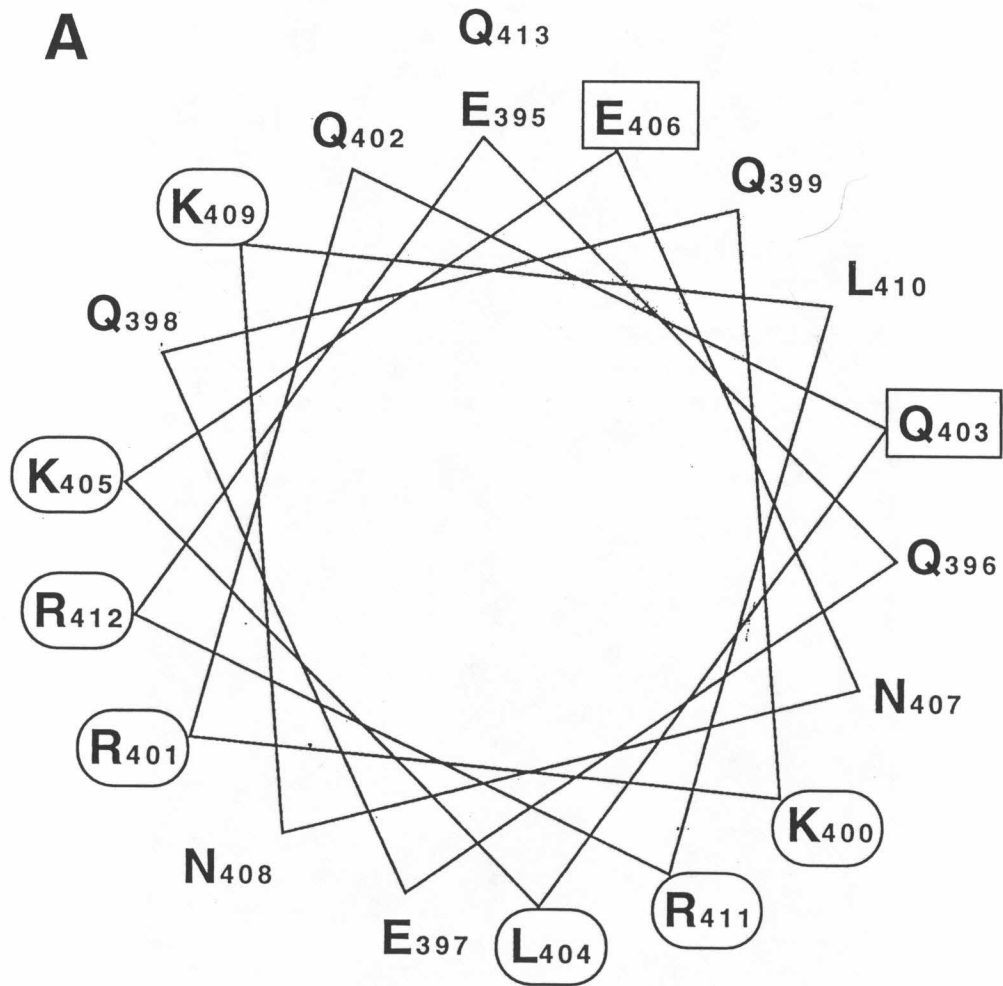
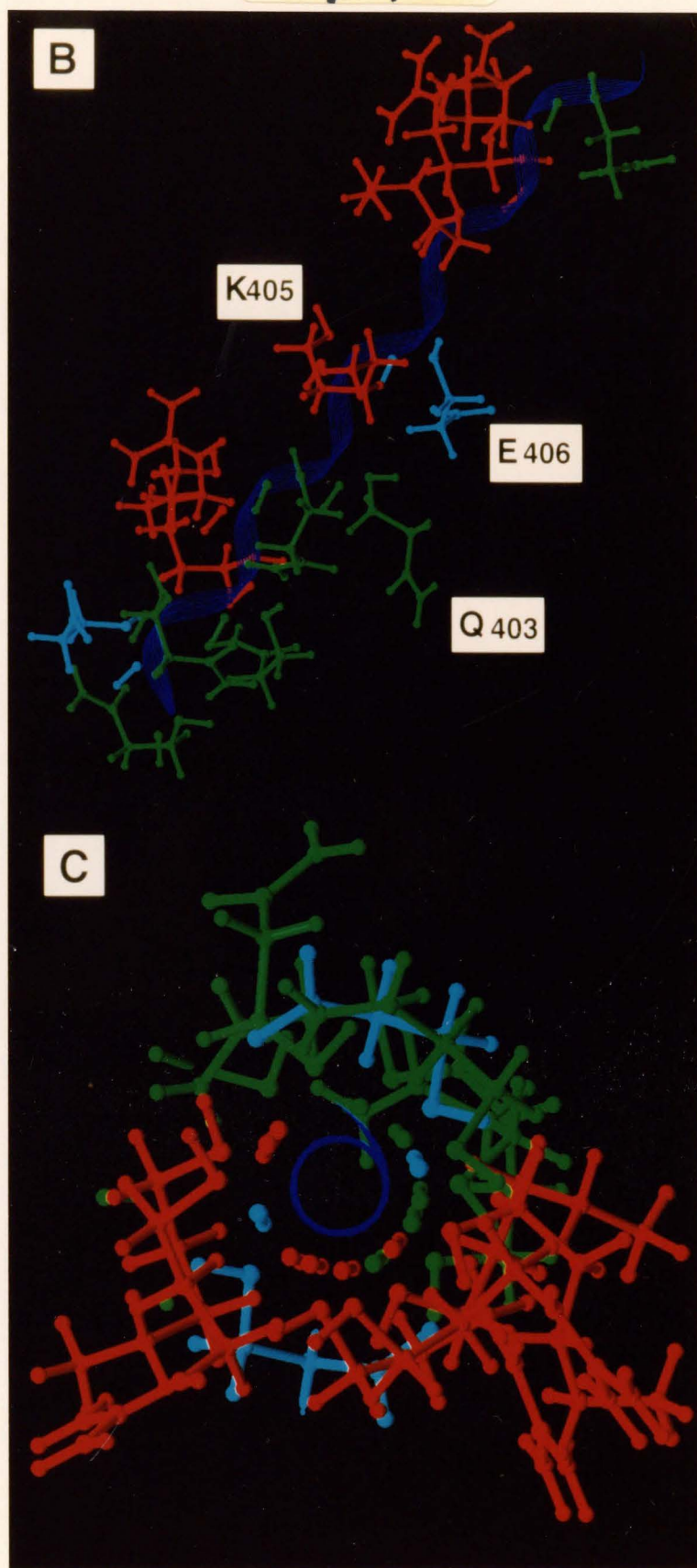


Fig. 7A





Chapter 3

DNA-binding and Oligomerization of *Drosophila* Heat Shock
Transcription Factor (HSF) Regulated by Its Subcellular
Localization: A Role for the Nuclear Localization Signal in
Regulating HSF Activity

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Key Words: Heat Shock Transcription factor; DNA binding; Nuclear
localization signal

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Abstract

The over expression of *Drosophila* HSF (dHSF) in SL2 cells resulted in constitutive DNA binding when examined in whole cell extracts. We examined several dHSF mutants under similar conditions and found that all of these mutants bound DNA constitutively. In addition, the over expression of all tested mutants, with one exception (see below), resulted in the activation of DNA binding of the endogenous HSF in the non-shocked cells.

Using an CuSO_4 inducible expression system, we found that over expression causes the nuclear entry of wild type and all tested mutants with one exception. It is shown that the deletion of a small domain required for nuclear localization (Zandi *et al.*, 1995) allowed the oligomerization and DNA binding of the HSF in cytosol of non-shocked cells. Single point mutations within this region that caused the disruption of the nuclear entry also resulted in the constitutive DNA binding in cytosol without heat shock. We found that a single point mutation within the NLS allows the constitutive nuclear entry and DNA binding when the dHSF is expressed at low levels, suggesting that cytoplasmic sequestration of dHSF is a primary step in the regulatory pathway. Cytoplasmic sequestration is saturable and can be overridden by over expression, leading to the interesting prospect that a cytosolic component may be responsible of sequestration.

Introduction

The activity of specific transcription factors can be regulated at several levels, including DNA binding and transcriptional activation (Keegan *et al.*, 1986; for a review see Ptashne, 1988). Regulation of DNA binding often is the result of cytoplasmic sequestration through association with other proteins (for a review see Baeuerle and Henkel, 1994). In the presence of an appropriate signal, the associated proteins dissociate from the transcription factor allowing it to enter the nucleus (Baeuerle and Baltimore, 1988). Both DNA binding and transcriptional activation can be regulated by the posttranslational modification of the factor by transient phosphorylation (for a review see Karin, 1994). These mechanisms can act either separately or together in response to external signals (for review see Hill and Treisman, 1995). For example, the activities of the NF- κ B and NF- κ B-related proteins are modulated by cytoplasmic sequestration. I- κ B and I- κ B-related proteins associate with NF- κ B and NF- κ B-related proteins in the cytoplasm and prevent the exposure of NF- κ B's nuclear localization signal (NLS) to the nuclear transport machinery (Baeuerle and Baltimore, 1988; Beg *et al.*, 1992; Beg and Baldwin, Jr., 1994; for a review see Baeuerle and Henkel, 1994). Upon stimulation of cells by appropriate agents such as cytokins, the inhibitor becomes phosphorylated and dissociates from NF- κ B which now can enter

the nucleus and bind to its DNA target. The phosphorylation of I- κ B induces its proteolytic degradation (Henkel *et al.*, 1993).

Another example in which the cytoplasmic sequestration is involved in the regulation of a transcription factor is the glucocorticoid receptor (Picard and Yamamoto, 1987). The GR is kept in the cytosol of the cell in association with heat-shock proteins (Sanchez *et al.*, 1985). Hormonal induction triggers the dissociation of the heat-shock proteins and the subsequent nuclear entry and dimerization of the GR, followed by DNA binding and transcriptional activation (Picard and Yamamoto, 1987). More recently, it has been shown that the activity of the Arabidopsis photomorphogenic repressor, cop1, is regulated by light (Von Arnim and Deng, 1994). Cop1 resides in the nucleus and suppresses photomorphogenic development (Von Arnim and Deng, 1994). Light deactivates cop1 by causing its relocalization to the cytoplasm (Von Arnim and Deng, 1994). The mechanism of the dark-light transition of cop1 is not yet clear.

Another interesting example of signal induced transcription factor activation is the heat-shock transcription factor (HSF). The transcriptional activity of the HSF from various organisms from yeast to man is regulated by heat and other stressors (for review see Morimoto *et al.*, 1992; Voellmy, 1994). HSF activation in insects and vertebrates is thought to include a monomer to trimer transition and, in the case of vertebrates, stress-dependent nuclear localization (Clos *et al.*, 1990; Baler *et al.*, 1993; Sarge *et al.*, 1993; for a recent

review see Voellmy, 1994). Studies on the localization of mammalian HSF have shown that the inactive HSF resides in the cytosol of non-stressed cells in a monomeric form (Baler *et al.*, 1993; Sarge *et al.*, 1993). This was demonstrated by a combination of cellular fractionation, western blotting and immunocytochemical microscopic analysis (Baler *et al.*, 1993; Sarge *et al.*, 1993). The inactive human HSF present in the cytosolic fraction has been shown to trimerize and bind DNA by *in vitro* heating (Mosser *et al.*, 1990). *In vivo*, the activated form of the HSF is found in the nuclear fraction (Baler *et al.*, 1993; Sarge *et al.*, 1993). In the case of *Drosophila* HSF (dHSF), the previously reported biochemical and cytological data contradict each other (Clos *et al.*, 1990; Westwood *et al.*, 1991). One report shows that the cytosolic fraction of non-stressed *Drosophila* Schneider line 2 (SL2) cells contain the vast majority of the dHSF in an inactive monomeric form similar to the vertebrate HSF (Westwood *et al.*, 1991). In the same report, however, it is shown by indirect immuno staining of non-shocked SL2 cells, as well as salivary glands of *Drosophila* embryos, that the dHSF is nuclear (Westwood *et al.*, 1991). This paradox has been explained to be the result of the presumptive loose association of the dHSF within the nuclei of non-shocked cells, and the subsequent leakage of the factor out of the nucleus during preparation of the cytosolic extract.

Despite the conserved function of the HSF among all organisms, there is little overall protein sequence homology. For example, all HSFs from yeast to

man bind efficiently to the highly conserved heat shock response elements (HSE), which consist of an array of nGAAn repeats; they only share about 30% amino acid homology in the DNA binding domain at the amino terminus (Sorger *et al.*, 1988; Wiederecht *et al.*, 1988; Clos *et al.*, 1990; Sarge *et al.*, 1991; Scharff *et al.*, 1990; Gallo *et al.*, 1991; Rabindran *et al.*, 1991; Schuetz *et al.*, 1991). A second region of homology includes a conserved heptad leucine-isoleucine repeat (LIR), which is responsible for the oligomerization of the factor and is adjacent to the DNA binding domain (Sorger and Nelson, 1989). Vertebrate, *Drosophila* and plant HSFs all contain a third homology region, which is a conserved LIR at their carboxy terminus (Clos *et al.*, 1990; Sarge *et al.*, 1991; Scharff *et al.*, 1990; Gallo *et al.*, 1991; Rabindran *et al.*, 1991; Schuetz *et al.*, 1991). The ScHSF does not possess this third homology region, and binds constitutively to DNA (Sorger *et al.*, 1988; Wiederecht *et al.*, 1988; Jakobsen and Pelham, 1988). The fact raises the interesting idea that the third LIR might be involved in the regulation of the stress regulated monomer to trimer transition observed in *Drosophila* and vertebrates.

Rabindran *et al.* (1993) mutated three conserved hydrophobic amino acids within the carboxy terminal LIR of the human HSF (hHSF) and expressed it transiently in human embryonic kidney 293 cells. They showed that the introduced mutations resulted in the constitutive trimerization and DNA binding of the hHSF in whole cell extracts. A similar result was reported in the same paper for the dHSF (Rabindran *et al.*, 1993). Transient expression of

a mutation in the dHSF which consisted of a deletion of the carboxy-terminal LIR region in SL2 cells resulted in constitutive oligomerization and DNA binding of the factor as assayed in whole cell extracts. In the same paper, however, it was mentioned that over expression of wild type hHSF in 293 cells resulted in the constitutive DNA binding (Ranidran *et al.*, 1993). In a more recent study, Zuo *et al.* (1994) analyzed the DNA binding activity of hHSF with deletions spanning the entire protein in *Xenopus* oocytes. They identified sequences within three LIRs of hHSF that apparently function to maintain the factor in the inactive, monomeric conformation. They proposed that the activation of hHSF might involve the transition from an intramolecular to an intermolecular triple-stranded coil-coiled structure.

In this report we have examined the effects of the over expression of dHSF on the regulation of the oligomerization, DNA binding and the subcellular distribution of the dHSF in SL2 cells. We found that over expression of wild type dHSF causes constitutive DNA binding when examined in whole cell extracts. We examined several dHSF mutants under similar conditions and found that all of these mutants bound DNA constitutively. In addition, the over expression of all of the mutants, with one exception (see below), resulted in the activation of DNA binding of the endogenous HSF in the non-shocked cells apparently by hetero-trimer formation.

To avoid problems associated with over expression, we utilized an expression system in which the level of expression could be controlled. Using this system we found that over expression causes the nuclear entry of wild type and all tested mutants with one exception. It is shown that the deletion of a small domain required for nuclear localization (Zandi *et al.*, 1995) also results in the oligomerization and DNA binding of the HSF in cytosol of non-shocked cells. Single point mutations within this region also caused the disruption of the nuclear entry as well as the constitutive DNA binding in the cytosol. We found that a single point mutation within the NLS allows the constitutive nuclear entry and DNA binding when the dHSF is expressed at low levels, suggesting that cytoplasmic sequestration of dHSF is a primary step in the regulatory pathway. Additionally, the NLS responds to stress when placed in the context of another protein (Zandi *et al.*, 1995), suggesting that it is a modular unit. Cytoplasmic sequestration is saturable and can be overridden by over expression, leading to the interesting prospect that a cytosolic component may be responsible of sequestration.

Results

DNA binding of dHSF as a function of temperature and subcellular localization

Drosophila SL2 cells were fractionated into cytoplasmic and nuclear fractions prior to and at various times after heat shock. The presence of dHSF in these fractions was monitored by Western blotting with a monoclonal antibody prepared against the recombinant form of dHSF (see Materials and Methods). In non-shocked cells, the dHSF is primarily localized in the cytoplasmic fraction (Figure 1A). Upon transferring the cells to 37°C, there is a progressive loss of the HSF from the cytoplasm with a concomitant entry into the nucleus. After 20 minutes of heat shock, the dHSF is found almost exclusively in the nuclear fraction. The ability of the dHSF to specifically bind DNA in the cytoplasmic and nuclear fractions was also examined. DNA binding was assessed by gel electromobility shift (GEMS) assays employing a HSE oligonucleotide and either nuclear or cytoplasmic extracts adjusted to have cell-equivalent amounts of protein. Specific DNA binding by the dHSF present in the cytoplasmic fractions was not observed, despite the fact that there is a large amount of HSF present in the cytoplasm in non-shocked cells (Figure 1B). The inability of the dHSF present in the cytoplasm to bind DNA is observed even in cells subjected to heat shock. The only form of dHSF that

is capable of specific DNA binding is that which is present in the nuclear fraction of heat shocked cells.

The kinetics of DNA binding and nuclear localization may at first appear slow; however, the time of the temperature shock is measured from the moment the cells are transferred to a 37°C incubator and not the time at which the cells are actually at 37°C. As it usually takes some time for the media to warm up to the normal heat shock temperature of 37°C by this procedure, the time course really represents a gradual increase in temperature. This is reflected by the absence of specific DNA binding and nuclear localization at the early time points. However, by 20 minutes almost all of the HSF is localized in the nucleus and capable of DNA binding (compare Figures 1A and 1B). These observations suggest that activation of DNA binding of the dHSF correlates with its nuclear entry in response to heat shock.

Over expression of HSF results in constitutive DNA binding

The initial system used to identify the region(s) involved in the regulation of HSF DNA binding consisted of transient transfection employing SL2 cells. Expression of the dHSF was driven by the *Drosophila* actin 5c promoter (see Material and Methods). SL2 cells were transfected with vector-only, 0.5, 1 and 5 mg of the plasmid encoding the wild type HSF (dH-WT). Twenty-four hours after transfection, the cells were washed to remove the

calcium phosphate, and were further grown at 25° C for another 24 hours. The cells were then split in half; one half was allowed to continue growth at 25°C and the other half was heat-shocked at 37°C for 20 minutes. Whole cell extracts (WCE) of these cells were prepared and the HSE binding activity monitored by GEMS. The transfection efficiency of the SL2 cells in these experiments was estimated to be around 30%. As depicted in Figure 2A (lane 1), the WCE from non-shocked cells transfected with vector-only DNA shows no HSE binding. Upon heat-shock, DNA binding increases significantly (lane 2). Cells transfected with dH-WT demonstrate a considerable amount of specific HSE binding at normal growth temperatures (lanes 3, 5, 7). The level of DNA binding increases as the amount of transfected plasmid increases (0.5 to 5 µg). The WCEs of the heat-shocked cells show greater HSE binding than the corresponding non-shocked extracts (lanes 4, 6, 8). These data suggest that over expression of HSF results in constitutive DNA binding under non-shocked conditions.

We have recently identified a nuclear localization signal in the dHSF (Zandi *et al.*, 1995). Since the data presented in Figure 1 suggests a correlation between subcellular localization and DNA binding activity, we examined the DNA binding of a dHSF mutant in which the NLS region, amino acids 393-420, was deleted (designated as mutant Δ NLS). The experimental conditions were identical to those described for the dH-WT except that 0.5 µg of plasmid

DNA was used to transfect the cells. As shown in Figure 2B the Δ NLS mutant binds DNA equally well under both non-shocked and heat-shocked conditions. The DNA binding of the endogenous HSF, however, appears to be properly regulated in these fractions. These data support the observed correlation between subcellular localization and the regulation of dHSF DNA binding.

Using similar assay conditions, we also examined the DNA binding activity of three other HSF mutants: Δ 3h in which the cLIR is deleted (amino acids 580-620); Δ oligo in which the oligomerization domain is deleted (amino acids 191-253) and Δ FI in which the amino acids 406 to 693 are deleted. The results of GEMSs of these experiments are shown in Figure 2C-E. Mutant Δ 3h binds DNA when derived from both non-shocked and heat-shocked cells (Figure 2C). It appears, however, that the level of DNA binding is greater in the heat-shocked extract compared to the non-shocked extract. As was the case with the full length dHSF, expression of Δ 3h also resulted in the apparent activation of DNA binding of the endogenous dHSF in non-shocked cells (Figure 2C). The Δ oligo protein shows no DNA binding activity when expressed in cells grown either at non-shocked or heat-shocked temperatures; nor did it activate DNA binding of the endogenous factor (Figure 2D). Expression of Δ oligo in these experiments was confirmed by western blotting (data not shown). Further analysis of Δ oligo showed that it is not capable of

multimerization (data not shown). The dHSF mutant AFl, in which the carboxy half of the NLS is removed, binds to the HSE equally well in extracts derived from both non-shocked and heat-shocked cells (Figure 2E). In this case the DNA binding activity of the endogenous dHSF appears to be regulated properly.

From the above observations it appears that over expression of the wild type dHSF or various deletion mutations of dHSF can cause misregulation of DNA binding for both the endogenous and exogenously introduced factor. The observation that Δ NLS and AFl mutants bind DNA constitutively but do not cause a misregulation of the endogenous dHSF's DNA binding suggest that the region 393 to 420 of the dHSF might play an important role in regulating DNA binding activity. Although this conclusion is supported by the data shown in Figure 1, it is still possible that the relatively high levels of dHSF expression in the above experiments might have resulted in activation of these factors even if they failed to activate the endogenous factor.

Expression of HSF in SL2 cells under the control of an inducible promoter

To overcome the problems associated with over expression of exogenous dHSFs, we have utilized an inducible expression system in which the dHSF constructs were placed under the control of the *Drosophila* metallothioneine promoter (Otto *et al.*, 1987). The levels of HSF expression are then regulated by the concentration of CuSO_4 as well as the length of time that CuSO_4 is

present in the media. To facilitate the separation of the exogenous dHSF from the endogenous factor, six histidines were introduced in frame at the amino termini as described in Materials and Methods. For each construct, SL2 cells were transfected with the appropriate expression plasmid along with the pAcoHygro (the bacterial hygromycin phosphotransferase gene cloned downstream of the actin 5C promoter, see Material and Methods). Hygromycin resistant cells were selected and stable cell lines were generated. Since it is known that high concentrations of heavy metal ions induce the heat-shock response (for a review see Morimoto *et al.*, 1992), we first determined the concentration range of CuSO_4 in which the activity of the endogenous dHSF is properly regulated. We found that concentrations of CuSO_4 up to 5 mM and 5 hours of incubation did not induce the dHSF to bind DNA (data not shown). In general, 1mM CuSO_4 provided sufficient levels of HSF expression for the purpose of these studies.

HSF expression was induced with 1mM CuSO_4 for one, two and three hours. At first, we determined the DNA binding activity and the subcellular distribution of the endogenous HSF in cells carrying the vector-only construct. As shown in Figure 3A, in cells induced with 1 mM CuSO_4 for the indicated times, the subcellular distribution (Figure 3A, western) and the DNA binding (Figure 3A, DNA) of HSF is similar to those cells which were not exposed to CuSO_4 . Western analysis using monoclonal antibodies prepared against the recombinant HSF shows (Figure 3A, lanes 1, 5, 10 and 13)

that the majority of the dHSF is present in the cytosolic extracts of non-shocked cells. It is important to note that in these fractions the dHSF does not bind the HSE (Figure 3A, DNA). In heat-shocked cells the dHSF is present largely in the nuclear fraction (lanes 4, 8, 12 and 16), although a small amount is present in the cytosolic fraction. As expected, the DNA binding activity is much greater in the nuclear fraction of the heat-shocked cells.

The oligomerization state of dHSF present in the cytosolic fraction of non-shocked cells, as well as those present in the nuclear fraction of the heat-shocked cells, was analyzed by gel filtration chromatography (Figure 2B, western). Cytosolic HSF migrates in the molecular weight (MW) range slightly above the 150 kDa marker, which indicates that the dHSF may be in a monomeric state. The nuclear form migrates in the MW range between 440-669 kDa, indicating a higher order of oligomeric form. The HSE binding activity of the gel filtration fractions was monitored as well, and the results are shown in Figure 2B (DNA part). Note that the low MW form of dHSF in the cytosolic fraction of non-shocked cells does not bind DNA. Oligomeric dHSFs in the nuclear extract of the heat-shocked cells avidly bind the HSE.

Next, we examined the subcellular distribution and the DNA binding activities of the HSF in the cytosolic and nuclear fractions of cells in which the expression of the dH-WT was induced with CuSO_4 for one, two, or three hours. In cells that are not induced with CuSO_4 , the HSF-DNA complex is seen only in the nuclear fraction of the heat-shocked cells (Figure 4A, DNA).

The subcellular distribution of the dHSF in these fractions is properly regulated (Figure 4A, western, lane 1 through 4). As the cells are induced with CuSO_4 for one, two and three hours, higher levels of HSFs are detected in the cytosol of the non-shocked cells (Figure 4A, western, lanes 5, 9, 13). Cytosolic fractions do not show a significant level of DNA binding activity. As the levels of dHSF increase, the factor is progressively detected in the nuclear fractions of the non-shocked cells (Figure 4A, western, lanes 7, 11, 15). This form of nuclear dHSF binds the HSE with high affinity as seen in the corresponding lanes in Figure 4A-DNA (1-Nu,h; 2-Nu,h; 3-Nu,h). There are two interesting points that should be noted here: One is that the detected amount of dHSF in the cytosol and nuclear fractions of the non-shocked cells at various levels of dHSF expression appear to increase simultaneously. The increased cytosolic level of dHSF, however, does not permit increased DNA binding. It seems that the ability of the cytosol to sequester HSF improves as the expression level of HSF increase. This raises the possibility of an auto-regulatory feedback mechanism where higher levels of dHSF might augment the synthesis or activity of putative cytosolic inhibitors. Secondly, it has been suggested (Westwood *et al.*, 1991) that dHSF is loosely associated with the nuclei in non-shocked cells. If this were the case, all of the overexpressed dHSF would be expected to be found in the cytosolic fractions in non-shocked cells. We observe essentially an equal distribution of dHSF between the nuclear and cytosolic fractions.

In the case of heat-shocked cells, normal heat-dependent nuclear entry of the expressed dHSF is observed (Figure 4A). There is a very small amount of dHSF in the cytosolic fractions of heat-shocked cells (Figure 4A; western, lanes 6, 10, and 14). The nuclear form of dHSF binds DNA with high affinity, as expected. In order to obtain a quantitative measure of differences in the DNA binding properties of the dHSF, all bands in GEMS were quantitated by a phosphor-imager and the results are presented in Figure 4B. From this analysis it is apparent that the levels of DNA binding in the nuclear fractions of the non-shocked cells becomes greater as the level of the HSF expression increases. After three hours of induction, the dHSE binding activities in the nuclear fractions of the non-shocked and the heat-shocked cells are similar. In order to compare the fold-induction of the DNA binding activities between the cytosol of the non-shocked and the nuclear of the heat-shocked extracts, the fold-induction for each time point was determined and plotted against the expression levels of dHSF (Figure 4C). The induction level increases rapidly between the time 0 (32 fold) and 1 hour (76 fold). After 2 hours, the expression of dHSF has increased and the induction level drops to a level similar to time 0. Between the time points 2 and 3 (25 fold) there is a further slight decrease of the fold-induction.

The pattern of the subcellular distribution of the HSF between the cytosol and the nuclear non-shocked cells (the increased presence of HSF in the nuclear non-shocked fractions as a function of increased level of its

expression), the constitutive DNA binding of HSF in the nuclear fraction of non-shocked cells and the assumption that the cytosol has a limited capacity for the sequestration of HSF suggest an inhibitory mechanism for HSF in the cytosol. Furthermore, if the nuclear entry of the dHSF is a regulatory step in this mechanism, it is important to investigate the role of the nuclear localization sequence (NLS) of the HSF in the regulation of HSF activity in more detail.

Role of the NLS in the regulation of DNA binding

We have recently learned that the nuclear entry of the dHSF is regulated by heat-shock. A bipartite NLS region on HSF was then identified (Zandi *et al.*, 1995). The deletion of amino acids 393 to 420 (construct Δ NLS in Figure 2B), which contains the NLS region, resulted in the disruption of nuclear entry under all conditions (Zandi *et al.*, 1995). The DNA binding and subcellular distribution of Δ NLS was analyzed under the same conditions as for dH-WT in Figure 4. The results of the DNA binding and western analysis of the subcellular fractionation for Δ NLS are shown in Figure 5. The western analysis shows that under normal or heat-shock conditions the Δ NLS remains in cytosol independently of its expression level (Figure 5A, western lanes 5, 6, 9, 10, 13 and 14). Surprisingly, the Δ NLS present in these fractions bind HSE with high affinity (compare the corresponding lanes of western to

those of DNA in Figure 5A). The cytosolic heat-shocked form of Δ NLS shows comparable DNA binding affinity as the one in the non-shocked cells. The Δ NLS present in the non-shocked and the heat-shocked extracts bind DNA even at low concentration after only one hour of CuSO_4 induction. The chart diagram Δ NLS (Figure 5B) illustrates that there is very little quantitative differences between the DNA binding affinities of Δ NLS in cytosolic non-shocked and heat-shocked extracts. These differences are shown in the fold-induction curve for Δ NLS in Figure 5C. Since the Δ NLS is always cytosolic, the fold inductions for Δ NLS were calculated for cytosolic fractions. Therefore, each point of this plot presents the fold-induction of DNA bindings between the cytosol of the non- versus heat-shocked cells. The fold-induction at the time 0 (cells which were not induced with CuSO_4) is 3.6 and it goes down to 2.7 by time point 1. By the time points 2 and 3 the fold-induction is 1. As it is obvious from the fold-induction values, the Δ NLS bind equally well both in the non-shocked and the heat-shocked conditions. It is important to note that the activity of the endogenous HSF is properly regulated in these extracts. The fold-induction of the endogenous HSF present in the cytosolic fraction of the non-shocked and the nuclear of the heat shocked cells at the time 0 was calculated to be about 32 which is comparable to those of the dH-WT. Taken together, these data further support the notion that subcellular localization of the dHSF determines the

state of the oligomerization and DNA binding activity. The data suggest also that the NLS region plays a crucial role in the regulation of both the oligomerization and DNA binding.

The role of the cLIR

It has been reported that the deletion of the conserved leucine-isoleucine repeat (cLIR) at the carboxy terminal region of HSF resulted in the constitutive oligomerization and DNA binding of the factor when tested in the WCE (Rabindran *et al.*, 1993). As we show here in Figure 2C the previously reported results might have been the effect caused by the over expression of the factor. We were interested to see whether the deletion of cLIR would result in constitutive DNA binding of HSF in the cytosolic fraction under various levels of protein expression. Therefore, $\Delta 3h$ (deletion of amino acids 520 to 620) was subjected to the same assay conditions as for dH-WT and results of the GEMS and western analysis are shown in Figure 6. The subcellular distribution of $\Delta 3h$ is properly regulated when the cells are induced with $CuSO_4$ for one and two hours (Figure 6A, western, lanes 5 through 12). When the cells were induced for three hours, a significant amount of the protein is detected in the nuclear fraction of the heat-shocked cells (lane 14 of Figure 6A, western). The cytosolic fractions of all non-shocked cells expressing $\Delta 3h$ show a little DNA binding activity. However,

there is a large increase in DNA binding when the $\Delta 3h$ is in the nuclear fractions (Figure 6A, DNA). The comparison of the detected $\Delta 3h$ in each fraction shown by western analysis (Figure 6A, western) to their corresponding DNA binding activities (Figure 6A, DNA) illustrate the fact that the DNA binding of $\Delta 3h$ is induced once the factor appears in the nuclear fractions. The chart diagram of $\Delta 3h$ (Figure 6B), which shows a quantitative comparison of the DNA binding activities of $\Delta 3h$ in various fractions, illustrates that the $\Delta 3h$ in the nuclear heat shocked binds 15-38 fold stronger to HSE compared to the $\Delta 3h$ in the cytosolic fraction of non-shocked cells (Figure 6B, $\Delta 3h$ compare CNs and NHs). This demonstrates the inducibility of $\Delta 3h$ in response to heat-shock. Similar to the dH-WT, the high level of $\Delta 3h$ expression results in the constitutive nuclear entry of some of the factor in the non-shocked cells (Figure 6A, western) and this form of the factor binds DNA with high affinity (Figure 6A, DNA). As for the dH-WT, a fold-induction curve for the $\Delta 3h$ was determined (Figure 6C). The fold-induction at the time 0 is 18 and it increases to 38 by the time 1. At the time point 2, the fold-induction returns to 15 and it goes further down to 8 by the time point 3. It is apparent that at each time point the DNA binding is induced by heat shock. Qualitatively, the shape of this fold-induction curve is similar to the one of the dH-WT; however, all of the fold-induction values of $\Delta 3h$ are lower

than those of the dH-WT by a factor of two. Nonetheless, when the course of the fold-induction curves of dH-WT and $\Delta 3h$ are compared, it becomes even more clear that the $\Delta 3h$ goes through similar regulatory pathway as to the dH-WT. Taken together, we conclude that the subcellular distribution and the DNA binding activity of the $\Delta 3h$ are regulated similarly to the one of the wild type HSF (dH-WT).

Comparison of the oligomerization states of dH-WT, $\Delta 3h$ and ΔNLS

The trimerization of HSF in response to heat-shock has shown to be an important step in the regulation of HSF's activity (Peteranderl and Nelson, 1992; Westwood and Wu, 1993; for review see Sorger, 1991; Voellmy, 1994). Here we analyzed the oligomerization state of the dH-WT, $\Delta 3h$ and ΔNLS by gel filtration. In these assays, for dH-WT and $\Delta 3h$, we employed the cytosolic non-shock and the nuclear heat-shock fractions of cells which were induced for three hours with $CuSO_4$. In the case of ΔNLS , the cytosolic non-shock and the cytosolic heat-shock fractions were assayed. Upon fractionation of extracts on a superdex 200 gel filtration column, the expressed HSFs were isolated from the fractions via Ni-agarose in the presence of 3M guanidine hydrochloride (see Materials and Methods). This step was taken to concentrate the HSFs in each fraction for western analysis and to isolate specifically the expressed HSFs. The results of the western analysis of these

assays are presented in Figure 7. The dH-WTs present in the cytosol of the non-shocked cells migrate in the MW range of 150 kDa consistent with the monomer form of HSF (Figure 7A NS panel lanes 7, 8, and 9). The dH-WTs in the nuclear fraction of the heat-shocked cells migrates faster at positions consistent with oligomeric form of the factor (Figure 7A, panel HS, lanes 2, 3, 4, and 5).

For the Δ NLS, the cytosolic fraction of the non-shocked cells was analyzed and as it is shown in Figure 7B, the Δ NLS migrates at the high MW range consistent with its oligomeric form (NS panel, western, lanes 2, 3, and 4). The DNA binding in these fractions was monitored as well. Figure 7B shows the HSE binding activities of these fractions (panel HS, DNA, lanes 2, 3, and 4). The chromatographic behavior of the Δ NLS in the cytosolic fraction of the heat-shocked cells is exactly like the one in the cytosolic fraction of the non-shocked cells (data not shown).

The Δ 3h present in the cytosol of the non-shocked cells fractionates at the MW ranges on the column consistent with the monomeric form of the protein (Figure 7C, NS panel western, lanes 6, 7, 8, and 9). The DNA binding activities in these fractions was monitored. As it is shown in Figure 7C (NS panel, DNA part), the Δ 3h in these fractions do not bind HSE. The Δ 3h in the nuclear fraction of the heat-shocked cells migrates at similar positions comparable to the dH-WT in the nuclear fraction of the heat-shocked cells.

The $\Delta 3h$ in these fractions binds the HSE with high affinity (Figure 7C, HS panel western and DNA, lanes 2, 3, 4, 5 and 5).

In summary, the analysis of the oligomerization states show that dH-WT exists in a monomeric form in the cytosol while the nuclear form is oligomeric. The deletion of the cLIR ($\Delta 3h$ construct) does not cause constitutive oligomerization of the HSF in the cytosol under non-shock condition. Thus the oligomerization of the $\Delta 3h$ is regulated similarly to the one of the wild type HSF. However, the deletion of the NLS region resulted in the constitutive oligomerization of the factor in the cytosol of the non-shocked cells. This suggests that the NLS plays a role in the regulation of the oligomerization of the dHSF.

Single amino acid substitutions in the NLS caused the constitutive oligomerization and DNA binding of the dHSF.

To investigate whether the constitutive oligomerization and DNA binding of ΔNLS is the result of a conformational change caused by the deletion of the NLS, we analyzed four mutant HSFs that contain amino acid substitutions in the NLS region. The generation of these mutations is described elsewhere (Zandi *et al.*, 1995). We examined an HSF that contains three amino acid substitutions within the NLS region. The substitutions are glutamine (Q) 399 to leucine (L), lysine (K) 400 to arginine (R) and leucine 404

to arginine. These mutations do not have a negative effect on the NLS function (Zandi *et al.*, 1995). We designate this mutated HSF as LRR. The LRR was subjected to the same assay system as dH-WT in Figure 4 and the results are depicted in Figure 8A. The subcellular distribution of LRR in the fractions of the cytosolic and nuclear of non-shocked and heat-shocked cells are comparable to those of the dH-WT (compare Figure 4A and 8A). LRR binds HSE only in the nuclear fractions (Figure 8A, DNA). The chart diagram LRR in Figure 8A shows the quantitative differences in the DNA binding activities among various fractions. This chart diagram is very similar to the one of the dH-WT. Next we tested NLS mutated HSFs in which the nuclear entry was disrupted as a result of the mutations. The substitution of lysine 405 to methionine (M), and leucine 404 to proline (P) each was shown (Zandi *et al.*, 1995) to result in the disruption of the HSF's nuclear entry. We designate these mutated forms of HSFs as K405M and L404P, respectively. The analysis of the subcellular localization and DNA binding of K405M and L404P are presented in Figure 8B and 8C. K405M is detected only in the cytosolic fractions of the non-shocked or heat-shocked cells (Figure 8B, western, lanes 5, 6, 9, 10, 13, and 14). The K405M in these fractions binds DNA with high affinity constitutively (Figure 8B, DNA). The quantitative comparison of DNA binding activities of K405M presented as chart diagram in Figure 8B shows that K405M binds HSE with similar affinities in non-shock and heat-shock conditions. The L404P was tested similarly and as it is

shown in Figure 8C, the subcellular distribution and DNA binding activities of this mutant are very similar to those of the K405M. The subcellular distribution and DNA binding activities of Δ NLS compared to those of K405M and L404P indicates that a properly functional NLS region is required for the proper regulation of HSF.

As it is shown elsewhere (Zandi *et al.*, 1995), the subcellular distribution of the dHSF is regulated by heat shock and NLS is responsible for this function. It is also found that the single substitution of glutamine at position 403 to leucine (Q403L) resulted in the constitutive nuclear entry of HSF. Here we tested the subcellular distribution and the DNA binding activities of Q403L. As it is shown in Figure 8D, Q403L is detected only in the nuclear fractions of non-shocked and heat-shocked cells (western, lanes 7, 8, 11, 12, 15 and 16). It is important to note that the intensity of bands seen in the cytosolic fraction of the non-shocked cells remain constant throughout the western figure (lanes 1, 5, 9, and 13). The DNA binding of Q403L seems equally strong in the nuclear fractions of non-shocked and heat-shocked cells (Figure 8D, DNA and chart Q403L).

Taken together it is apparent that a properly functional NLS is necessary for the regulation of the HSF activity in response to heat shock.

The oligomerization states of LRR, K405M, L404P and Q403L were analyzed by gel filtration. For LRR, the cytosolic non-shock fraction of the three hour CuSO_4 induction was compared to the corresponding nuclear

heat-shock one. The western analysis of fractionated LRRs is shown in Figure 9A. The cytosolic LRR migrates in the MW range consistent with the monomer form (Figure 9A, NS panel lanes 5, 6, and 7). The heat-shocked form of LRR migrates in the MW range consistent with the oligomeric form of the protein (Figure 9A, HS panel, lanes 2, 3, and 4). For K405M and L404P, the oligomerization states of proteins present in the cytosolic fractions of non-shocked and heat-shocked cells were compared. K405M and L404P migrate in the MW ranges consistent with the oligomeric state of these factors in both non-shock and heat-shock fractions (Figure 9B and 9C, lanes 2, 3, and 4 of NS and HS panels). These observations are consistent with their constitutive DNA binding activities. For Q403L, the fractions of nuclear non-shocked and heat-shocked were analyzed and as it is depicted in Figure 9D in both non-shocked and heat-shocked conditions, Q403L migrates in the high MW range as oligomers (Q403L, lanes 2, 3, and 4 of NS and HS panel). This data indicates that once HSF enters the nucleus, even under normal condition, it oligomerizes.

To confirm that the biochemical fractionation data shown above reflect the actual subcellular distributions of these mutants in cells, the subcellular distribution of LRR, K405M, L404P and Q403L were examined by immunolocalization (Figure 10). When the expression of LRR was induced for one hour, the protein was localized exclusively in the cytoplasm (Figure 10A-1). However, when the expression of LRR was induced for two or three

hours, the staining pattern for LRR was distributed all over the cell (Figure 10A-2 and 3). After heat shock, in cells in which LRR was induced for one hour, the protein was exclusively nuclear (Figure 10A, HS). The expression of K405M and L404P were induced for one hour and in both cases these factors remain cytosolic under non- and heat-shock conditions (Figure 10B and 10C). The Q403L protein even at low level of expression (one hour induction) is nuclear in the non-shocked and heat shocked cells (Figure 10D). Taken together, the immunolocalization studies of these mutants agree with the biochemical fractionation data.

Discussion

In this study we investigate the regulation of the oligomerization and DNA binding of the dHSF in *Drosophila* SL2 cells. We show that the dHSF is present in the cytosolic low salt fraction of the non-shock cells in a monomeric form that is incapable of DNA binding. In heat-stressed cells, the factor is present in the nuclear fraction in a trimeric state and binds DNA with high affinity. When the wild type HSF (dH-WT) was expressed in the SL2 cells as a function of its expression level, a portion of HSFs entered the nucleus constitutively in non-shock cells. These HSFs bound HSE with an affinity similar to the HSFs in the nuclear heat-shock fraction. Previously, using cytological methods, it has been reported that the dHSF is nuclear in the non-shock cells (Westwood *et al.*, 1991). However, by biochemical fractionation, HSF was found in the cytosolic low salt fraction of the non-shock cells (Clos *et al.*, 1990; Westwood *et al.*, 1991). This contradiction has been explained to be a result of the loose association of the dHSF with the nucleus in the non-shock cells. A loose association of the factor with the nucleus in this condition would suggest that, independent of the amount of HSF in the cell, all of HSF would be present in the cytosolic low salt fraction. However, as we demonstrate here, when the dH-WT was expressed at various levels, the cytosolic low salt fraction became saturated with HSF and as a function of expression level, the factor appeared in the nuclear fraction of the non-shock cells. This indicates that the factor enters the nucleus once its

amount in the cytosol exceeds a certain level. Although HSFs were present at high levels in the cytosolic fraction, they were found to be monomeric and did not bind HSE. On the other hand, even present at lower amounts, HSFs in the nuclear fractions of these cells bind HSE. These observations do not support the idea of a loose association of HSF with the nucleus in the non-shock cells. On the contrary, our results show that depending on the cellular stress condition, HSF has a fixed location in the cell. Furthermore, the subcellular location of the factor seems to be crucial for its state of activity. Those populations of HSFs which are sequestered from the nuclei are found to be in the monomeric, inactive form in the cytosol, and they do not bind HSE. The population of HSFs, which enter the nucleus, however, bind DNA and are oligomeric. The fact that the oligomerization state of HSF depends on its subcellular residency suggests that mechanisms of both the cytosolic retention and the maintenance of the monomeric form are either the same or very closely interconnected.

The fact that the over expression of HSF resulted in the constitutive nuclear entry of some of the factor suggests that HSF is under negative control in the cytosol. A cytosolic inhibitory factor(s), whose amount is limited, may associate with HSF in order to retain and maintain it in the inactive monomeric state in the cytosol of the non-stressed cells. Two additional observations support this idea. First, the HSF in the cytosolic extract of the non-shock SL2 cells was activated by *in vitro* heat-shock

(Westwood *et al.*, 1991; our unpublished observations). Secondly, when cytosolic fraction of the non-shock cells was fractionated on a gel filtration column, monomeric HSF present in the low (150 kDa) MW migrating fractions was activated to oligomers by *in vitro* heat shock (our unpublished data). From these, one can also conclude that the inhibitory mechanism itself is sensitive to heat or generally stress, and the dissociation of inhibitor(s) from HSF occurs by the raise of temperature. It is also possible that the mechanism of this dissociation process include heat regulated dephosphorylation, phosphorylation or other kind of covalent modifications of HSF, inhibitor or both. This would suggest, however, that the enzymatic activity necessary for the release of HSF from inhibitor would co-migrate with HSF on the gel filtration column.

The sequestration capacity of the cytosol for HSF increases with the increasing level of the HSF expression. This might suggest that HSF itself is involved in the regulation of the expression of some or all components of the inhibitory mechanism. Several reports have suggested that heat shock proteins might be involved in the regulation of HSF activity (Abravaya *et al.*, 1992; Mosser *et al.*, 1993; for a review see Voellmy, 1994); and since HSF induces the expression of hsps, thus HSF may autoregulate its activity. It is possible that some of known or yet undiscovered hsps are integral components of the putative inhibitory mechanism.

Sequences of HSF involved in the regulation of oligomerization state and DNA binding

Since only trimers of HSFs bind HSE with high affinity, it is obvious that the trimerization of HSF is a prerequisite step for the DNA binding. The mechanism of the monomer to trimer transition of HSF, however, is not yet fully understood. In one report, it was shown that the deletion of cLIR in human and *Drosophila* HSF resulted in the constitutive oligomerization in case of each factor (Rabindran *et al.*, 1993). However, in those experiments, two possible regulatory steps were not considered: first, the effect of the over expression of HSF on its regulatory mechanism, and second, the subcellular distribution of HSF that would play a role in the regulation of the oligomeric state of the factor. According to their observations in that report, authors suggest that an intramolecular interaction between cLIR and other regions of HSF (oligomerization domain specifically) would be the mechanism for the maintenance of the monomeric state of HSF (Rabindran *et al.*, 1993). However, it is not clear how the proposed mechanism would explain the fact that the full length recombinant HSF is oligomeric under all conditions. Here we first showed that when the HSF's deletions or wild type HSF are expressed in cells and their DNA binding is assayed in a whole cell extract, constitutive DNA binding was observed for all of them. As we then took the effect of the over expression into the consideration and, in addition, analyzed cell fractions instead of whole cell extracts, we were able to dissect the active

and inactive populations of expressed dH-WT (see above) or $\Delta 3h$ in the cell fractions. Our data clearly demonstrates that the $\Delta 3h$ goes through the same regulatory pathway as the wild type HSF in regard to the regulation of the oligomerization state and DNA binding. When $\Delta 3h$ was expressed in cells, depending on its expression level, similarly to the dH-WT, a fraction of $\Delta 3hs$ enter the nucleus and bind DNA under non-shock condition. The retained fraction of $\Delta 3hs$ in the cytosol remain as monomers and do not bind HSE. These observations show clearly that deletion of cILR does not cause the constitutive oligomerization and DNA binding of the dHSF. At this point, however, we cannot rule out that this may be a *Drosophila* specific mode of regulation. Since there is only a limited number of amino acid sequence homology between vertebrate and *Drosophila* factors, it is possible that because of the differences in the living's conditions, the processes involved in the activation of HSFs in various organisms have diverged throughout the evolution as it has in the case of scHSF.

In a more recent study it was shown, using a heterologous system, that there are at least three LIRs (LZ1, LZ2 and LZ3) that are involved in the regulation of the oligomerization of the hHSF (Zuo *et al.*, 1994). At this point we cannot compare our observations of the dHSF regulation in the SL2 cells with those of the hHSF observed in the *Xenopus* oocytes at the oocytes specific temperatures. It would be interesting to see as to whether mutations in the LZ regions of hHSF which caused the constitutive oligomerization of

the factor in the *Xenopus* oocytes would have similar effect on the regulation of the factor in the fractionated human cells growing at their natural temperature.

The analysis of the wild type dHSF in SL2 cells suggested a close connection between the subcellular localization and the oligomerization state of the factor. As we analyzed this connection further, we found that the deletion or loss-of-function mutations in the NLS resulted not only in the disruption of the nuclear entry, but also constitutive oligomerization and high affinity HSE binding in the cytosol of the non-shock cells. This observation suggests that the NLS plays a central role in the activation process of HSF. One could, however, argue that the constitutive oligomerization and DNA binding of Δ NLS could have been the result of a random conformational change(s), simply caused by the deletion of the NLS, which has prevented the HSF from entering the nucleus. The single amino acid substitutions (K405 to M) within NLS, however, caused the disruption of the nuclear entry and resulted, at the same time, in the constitutive activation of HSF similar to the effect caused by the deletion of NLS. Thus the constitutive oligomerization of Δ NLS is not because of a simple random conformational change, but it is rather related to a mechanism regulating the activation process of the HSF. Furthermore, the fact that the NLS is modular was enforced when the single change of glutamin 403 to leucine resulted in the constitutive nuclear entry of HSF, and this was independent of the expression

level of the factor. The DNA binding of this HSF mutant (Q403L) is constitutive. It is very interesting that the oligomerization state of the dHSF is mainly controlled by its subcellular localization and the proper functioning of its NLS is necessary for this regulatory mechanism. How can this mechanism be explained? It is possible that NLS regulates the oligomerization state of the dHSF via a direct interaction with other regions on HSF, e.g., oligomerization domain, or the interaction with an accessory factor which would prevent the HSF from nuclear entry and oligomerization at the same time. The fact that the affinity purified recombinant HSF is oligomeric under all conditions would rule out the likelihood of a functional intramolecular interaction of NLS with other regions on HSF as the mechanism for the monomer formation under non-stress condition. On the other hand, the idea of an inhibitory factor is rather favored here because the over expression of the wild type HSF resulted in the constitutive nuclear entry of the factor suggesting the out titration of a regulatory factor(s). Since only deletion or mutations in the NLS region caused constitutive oligomerization of the HSF in the cytosol, it is likely that the above mentioned putative inhibitory factor(s) interacts with the NLS. This interaction would not only mask the NLS from the exposure to the nuclear targeting proteins, but also maintain HSF in a monomeric conformation. It is also possible that the putative factor would interact with other regions of HSF in addition to the NLS. However, it seems that the only relevant interaction

regarding the regulation of the oligomerization occurs only through the NLS. In addition we did not find any other regions on HSF, whose removal would cause constitutive oligomerization in the cytosol or constitutive nuclear entry.

The composition of the amino acid sequence of the NLS indicates that it belongs to the bipartite NLS family (Dingwall and Laskey, 1991). This motif consists of a short stretch of two or three basic amino acids (K and R), followed by any ten amino acids and a second short stretch of basic amino acids (usually a combination of 2 or 3 Ks and Rs). The Chou-Fasman hydrophobicity-hydrophilicity prediction of the entire dHSF sequence suggests a strong probability of an α -helical secondary structure for the NLS region. The computer modeling analysis of this sequence as α -helix shows that it is an amphipathic helix (Zandi *et al.*, 1995). One face of the helical cylinder is covered with Ks and Rs resulting in a positively charged surface. The other side of the helical cylinder is covered with Qs and Es resulting in rather a negatively charged or Q-E rich face. Since substitutions of the Ks or Rs on the K-R rich face of the NLS resulted in the disruption of the nuclear (Zandi *et al.*, 1995) entry and constitutive oligomerization of HSF in the cytosol, and the substitution of Q403 on the Q-E rich face resulted in the constitutive nuclear entry, it is conceivable to suggest that the Q-E rich side of the NLS plays a modular role for the function of the NLS. The K-R rich side would be the binding site for a component of the nuclear targeting machinery

(see Figure 11), and the Q-E rich face of helix is the recognition site for the putative inhibitory factor. However, for a stable and efficient binding, the inhibitory factor still needs the interaction with the Ks and Rs on the K-R rich face. This would explain why mutations on the K-R rich side resulted in the constitutive oligomerization (the efficient binding of the inhibitor has been impaired because of the mutations). From this scenario one could come to the conclusion that the putative cytoplasmic inhibitory factor and a component of the nuclear targeting machinery compete for the binding site on the NLS (see Figure 11). The heat-stress can change the equilibrium of the competition in favor of the nuclear targeting factor.

According to our model (Figure 11) and the fact that the recombinant HSF is constitutively oligomeric, it is likely that the oligomerization state of HSF in the higher eukaryotes is regulated by the modulation of an accessory factor(s) and not HSF itself. The identification of this factor(s) would allow one to obtain information about the heat-stress signal transduction pathway that is upstream of HSF. This would bring us one step closer to identification of the receptor that senses the changes of temperature in the cell.

Materials and Methods

Plasmids and DNA constructions

All plasmids and DNA constructs used for this study are described in Zandi *et al.* (1995).

Cell culture, stable and transient transformation

The *D. melanogaster* Schneider 2 cell line (SL2) (Schneider, 1972) were maintained in SL2 cell culture media (GIBCO) supplemented with 10% heat treated (at 65° C for 30 min) fetal bovine serum (Hy-clone) and 100U/ml penicillin and 100U/ml streptomycin. Cells were grown in Corning flasks at 25°C (non-shocked). For DNA transfection, 3×10^6 cells were seeded in 3 ml of medium on a 30 mm tissue culture dish and transfected using calcium phosphate precipitates (Ausubel *et al.*, 1992) of cocktail of 2 plasmids at indicated amounts along with 10 µg of pBluescript plasmid as carrier DNA. The cotransfection cocktail contained a mixture of 5 µg of the vector that contained the gene of interest and 1 µg of the vector that contained the selectable marker pPacHyg (Zandi *et al.*, 1995). At 15-18 hr post-transfection cells were spun down, washed once with 1x PBS containing 2 mM EDTA, and once with the complete medium, and resuspended in 3 ml of fresh medium. After 4 days, cells were spun down and resuspended in selective medium

containing hygromycin B (200 $\mu\text{g}/\text{ml}$). Selective medium was replaced every 5 days, and stably transformed polyclonal cell populations were isolated after 3 or 6 weeks of selection with hygromycin B. Hygromycin B was maintained routinely in the media at all times after selection.

For transient expression, cells were transfected as above, except no vector containing a selective marker gene was included in the transfection mixture and the amount of the DNA used for transfection is indicated in the figures. After transfection, cells were washed as above and grown for 2 days before harvesting them for experiments.

Cells were heat shocked at 37° C for 30 min on the tissue culture dish by transferring the plates to a 37° C incubator, or in an Eppendorf tube on a 37° C heat block, prior to preparation of extracts.

Preparation of mini whole, cytosolic and nuclear cell extracts

The cytoplasmic and nuclear extracts were prepared by modifications of the procedure described by (Schreiber *et al.*, 1988): 1.5 ml of the cell suspension (3×10^6 cells/ml) were centrifuged for 2 min. at 3000 rpm and resuspended in 1 x PBS for a wash. Cells were spun down at 3000 rpm and resuspended in 150 ml hypotonic buffer (A) containing 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2.5 mM DTT, 5 mg/ml Leupeptin and 1.2 mM PMSF and incubated on ice for 5 min. Then 150 ml of buffer A containing 0.5% of NP-40 was added and vortexed for 10 seconds. The mixture were

centrifuged at 14,000g for 2 minutes. The cytoplasmic supernatant was transferred into a new Eppendorf tube and nuclear pellet was washed once with 100 ml of 0.5% NP-40 in buffer A. Each nuclear pellet was resuspended in 200 ml of buffer C consisting of 20 mM HEPES pH 7.9, 25% glycerol, 0.8 M NaCl, 100 mM KCL, 0.01% triton X100, 1 mM EDTA, 1 mM EGTA and DTT/Leupeptin/PMSF as above. Samples were vortexed and incubated on ice for 15 minutes, and then centrifuged at 14,000g for 5 minutes. The supernatant is the nuclear extract.

The whole cell extracts were prepared from the same number of cells as above. After cells were washed with 1 x PBS as above, pellet cells were resuspended in 300 μ l of the C buffer and vortexed for 10 second followed by incubation on ice for 15 min. The mixture was then spun down at 14,000g for 10 min and the supernatant was used for the protein analysis.

CuSO₄ induction

We have analyzed and determined the optimum induction procedure for the Mtn promoter. Cells were induced routinely by addition of 1 mM of CuSO₄ to the culture medium for indicated times in figures.

Protein analysis: gel electromobility shift assay, western blot

8 ml of the cytoplasmic and 2 ml of the nuclear extracts were used for the gel electromobility shift assay. The differences in the salt concentrations of

cytosolic and nuclear extracts were adjusted by adding 2 μ l of buffer C to the gel shift reaction of the cytosolic extract, and adding 8 μ l of the buffer A to the gel shift reaction of the nuclear extract. In general the binding reactions (30 μ l) contain 0.2-0.5 ng (30000 c.p.m.) of a radiolabeled double stranded HSE oligonucleotide (seq: 5'GCGCGCCTCGAATGTTTCGCGAAAAGA3'), 1 μ g poly[(dA)(dT)], 1 μ g poly[(dI)(dC)], 0.5 μ g salmon testis DNA, 4% Ficoll, 20 mM HEPES pH 7.9, 50 mM NaCl, 20 mM KCl, 1 mM EDTA, 1 mM DTT and 0.25 mg/ml bovine serum albumin (BSA, Boehringer Mannheim) and extracts as described above. The samples were incubated on ice for 30 min. 15 μ l of the mixture was loaded on a 4% polyacrylamide gel (30:1 acrylamide:BIS acrylamide) and electrophoresed for 2-3 hr. The gel was usually pre-run for 30 min at 20°C at 12 V/cm in 0.25-X TBE. After electrophoresis the gel was dried and exposed to X-ray film or phosphorimager screen (Molecular Dynamics).

To the remaining 15 μ l of the gel shift reactions were separated on an 8% SDS-PAGE and transferred to a nitrocellulose membrane. After blocking the nitrocellulose with 3% (w/v) Carnation non-fat dry milk, the blot was probed with a 1:1000 dilution of a hybridoma supernatant containing anti-Drosophila HSF. Primary antibodies were visualized By ECL (Amersham) method as recommended by the vendor.

Gel filtration chromatography

For analytical gel filtration chromatography, 300 μ l of cytosolic extract or 100 μ l of nuclear extract was loaded on a superdex 200 HR 10/30 (0.5x30 cm, Pharmacia) column that was equilibrated in the buffer NPO [150 mM NaCl and 50 mM Na₂HPO₄ (pH 7,0)]. The column was run at 0.5 ml per minute flow rate. The void volume of this column is about 6 ml. Oligomeric HSF elutes between 6.5 and 8 ml, and monomeric HSF elutes between 8.5 and 10 ml. Fractions of 0.5 ml or 1 ml were collected. Proteins in each fraction were either TCA precipitated or, in the case of histidin-tagged proteins, they were concentrated on Ni-agarose beads (QIA Gene). Concentrated proteins were separated on SDS-PAGE, and dHSF was visualized by western blotting.

Cell staining and immunofluorescence was carried out as is described in Zandi *et al.* (1995).

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Figure legends

Figure 1. Distribution and DNA binding activity of *Drosophila* HSF in cytoplasmic and nuclear fractions of SL2 cells as a function of heat shock. C indicates that the cytoplasmic fraction was used, and N indicates that the nuclear fraction was used. The heat shock times in minutes on top of each figure refers to the time at which the cells were transferred to a 37°C incubator. **A.** Western blot analysis of HSF's distribution. Each lane contains an equivalent amount of protein on a cell number basis. Proteins were separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose. The nitrocellulose sheet was probed with a monoclonal antibody prepared against bacterially produced *Drosophila* HSF. Molecular weight markers (in kD) are indicated alongside the gel. dH refers to *Drosophila* HSF purified from heat shocked *Drosophila* Kc cells. **B.** Comparison of the specific DNA binding activity of the *Drosophila* HSF in crude cytoplasmic and nuclear extracts of nonshocked and heat shocked SL2 cells by gel electromobility shift assay. The arrowhead indicates the position of the HSF-HSE complex.

Figure 2. DNA-binding analysis of constitutive expressed full length (A) and deletion mutants (B-E) of *Drosophila* HSF in SL2 cell. The DNA-binding of HSF and its derivatives were analyzed in the whole cell extracts of non-

shocked (N) and heat-shocked (H) SL2 cells transfected with full length dHSF (dH-WT) or deletion mutants (B-E). Cells were heat shocked for 30 minutes at 37°C as indicated in legend of Figure 1. The dHSF and its derivatives are schematically represented by stippled boxes on the left side of their corresponding data of gel shift assay. Numbers at the top of the first box (A) indicate the amino acid positions. The letters on the solid line at the top indicate the approximate functional domains of the dHSF. DNA, DNA-binding domain; OLIGO, oligomerization domain; NLS, nuclear localization domain; L-I, leucine-isoleucine repeat region. Numbers at the top of the pictures of autoradiogram indicate the amount of expression vector in μg that was used to transfect SL2 cells. For A the data of 0 to 5 μg transfections are shown and for B-E the data for 0.5 μg are shown. **A.** dH-WT, full length wild type *Drosophila* HSF. **B-D.** Δ indicates an internal deletion; numbers in parenthesis indicate the internally deleted region. **E.** N refers to the first amino acid of dHSF and 405 refers to last amino acid in this deletion.

Figure 3. Effect of CuSO_4 on DNA-binding, subcellular distribution, and oligomerization state of dHSF. *Drosophila* SL2 cell line stably transfected with pMt vector-only were incubated with 1mM CuSO_4 for indicated times in hours. **A.** DNA-binding activity (DNA) and subcellular distribution (western) of endogenous HSF were analyzed in the cytosol (C) and nuclear (Nu) extracts of the nonshocked (n) and heat shocked (h) cells. The top arrowhead on left

indicates the position of the HSF-HSE complex and the bottom one indicates the free HSE. **B.** Oligomerization state of endogenous HSF in cytosolic nonshocked (NS) and nuclear heat shocked (HS) extracts of cells incubated with CuSO_4 for three hours were analyzed by gel filtration on a superdex 200 HR 10/30 column. Fractions of 1 ml were collected and analyzed by western blot (western). The elution profiles are shown. The approximate elution positions of protein standards are indicated at top of elution profiles: thyroglobulin (MW 669,000), ferritin (MW 440,000), human IgG (MW 150,000). The DNA-binding of dHSF in gel filtration fractions was examined by gel shift (DNA).

Figure 4. Effect of expression level of wild type dHSF (dH-WT) on its subcellular distribution and regulation of DNA-binding. **A.** *Drosophila* SL2 cell line carrying stably integrated cDNA encoding dHSF under control of *Drosophila* metallothioneine were incubated with 1 mM CuSO_4 for indicated times in hours. DNA-binding activity (DNA) and subcellular distribution (western) of endogenous HSF was analyzed in the cytosol (C) and nuclear (Nu) extracts of the nonshocked (n) and heat shocked (h) cells. **B.** Quantification of DNA-binding activities of dH-WT presented in chart diagrams. CN, cytosol nonshock; CH, cytosol heat shock; NH, nuclear nonshock; NH, nuclear heat shock. **C.** Fold induction curve of dH-WT. Fold

induction for each time point was determined by dividing of band intensity of NH by CN.

Figure 5. Analysis of the DNA-binding activity and subcellular distribution of Δ NLS, a dHSF mutant in which the nuclear localization is deleted (see figure 2), in stably transformed SL2 cell. **A, B, and C.** The descriptions of these figures are exactly as figures 4A-C except the fold inductions of Δ NLS show the ratio of CH/CN.

Figure 6. Analysis of the DNA-binding activity and subcellular distribution of Δ 3h, a dHSF mutant in which the L-I region is deleted (see Figure 2), in stably transformed SL2 cell. **A, B, and C.** The descriptions of these figures are exactly as figures 4A-C.

Figure 7. Analysis of oligomerization state of expressed wild type dHSF (dH-WT), Δ NLS, and Δ 3h by gel filtration. Oligomerization state of dH-WT (**A**) and Δ 3h (**C**) in cytosolic nonshocked (NS) and nuclear heat shocked (HS), and Δ NLS (**B**) in cytosolic nonshocked (NS) of the extracts of cells incubated with CuSO_4 for three hours were analyzed by gel filtration on a superdex 200 HR 10/30 column. Fractions of 0.5 ml were collected and analyzed by western blot (western). The elution profiles are shown. The approximate elution positions of protein standards are indicated at top of elution profiles:

thyroglobulin (MW 669,000), ferritin (MW 440,000), human IgG (MW 150,000). The DNA-binding of Δ NLS and Δ 3h proteins in gel filtration fractions was examined by gel shift (DNA).

Figure 8. Analysis of the DNA-binding activity and subcellular distribution of dHSFs containing triple or single point mutations in stably transformed SL2 cell. The general descriptions of these figures are exactly as figure 4. In **A** LRR indicates the dHSF mutant in which amino acid residues glutamine (Q) 399, lysine (K) 400, and leucine (L) 404 are replaced by leucine, argenine, and argenine respectively. In **B-D** data of dHSF containing single point mutations are presented. Mutation in each case is indicated by single letter code of amino acids. The first letter indicates the wild type residue, the number shows the position, and the last letter indicates the mutant residue.

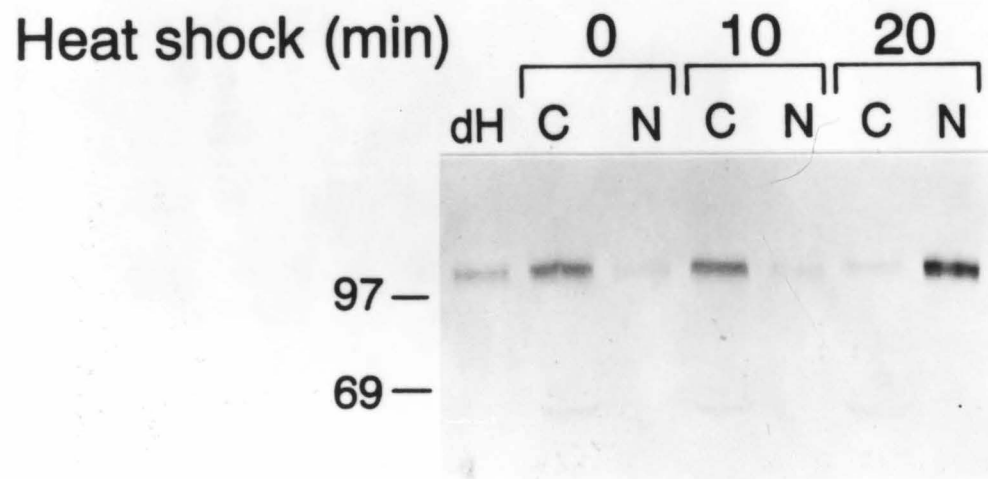
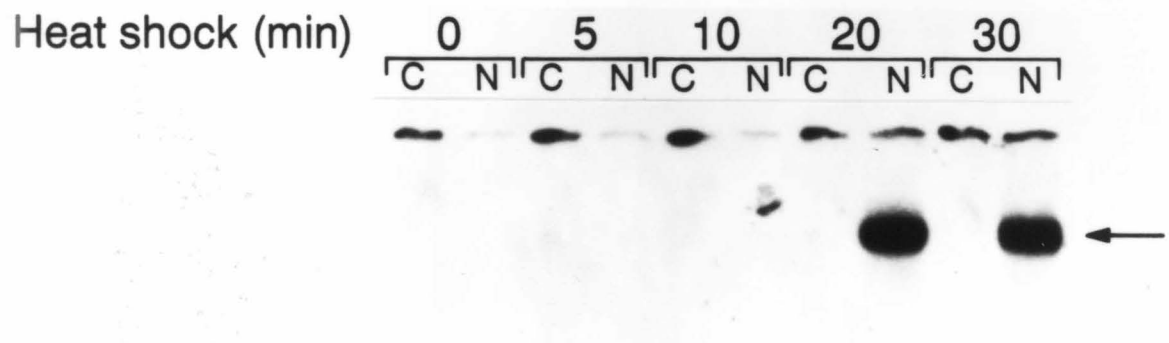
Figure 9. Analysis of oligomerization state of expressed dHSFs containing triple and single point mutations by gel filtration. Oligomerization state was analyzed for LRR (**A**) and Q403L in extracts of cytosolic nonshocked (NS) and nuclear heat shocked (HS), and for K405M (**B**) and L404P (**C**) in extracts of cytosolic nonshocked (NS) and cytosolic heat shock (HS) of cells incubated with 1 mM CuSO₄ for three hours by gel filtration on a superdex 200 HR 10/30 column. Fractions of 1 ml were collected and analyzed by western blot (western). The elution profiles are shown. The approximate elution

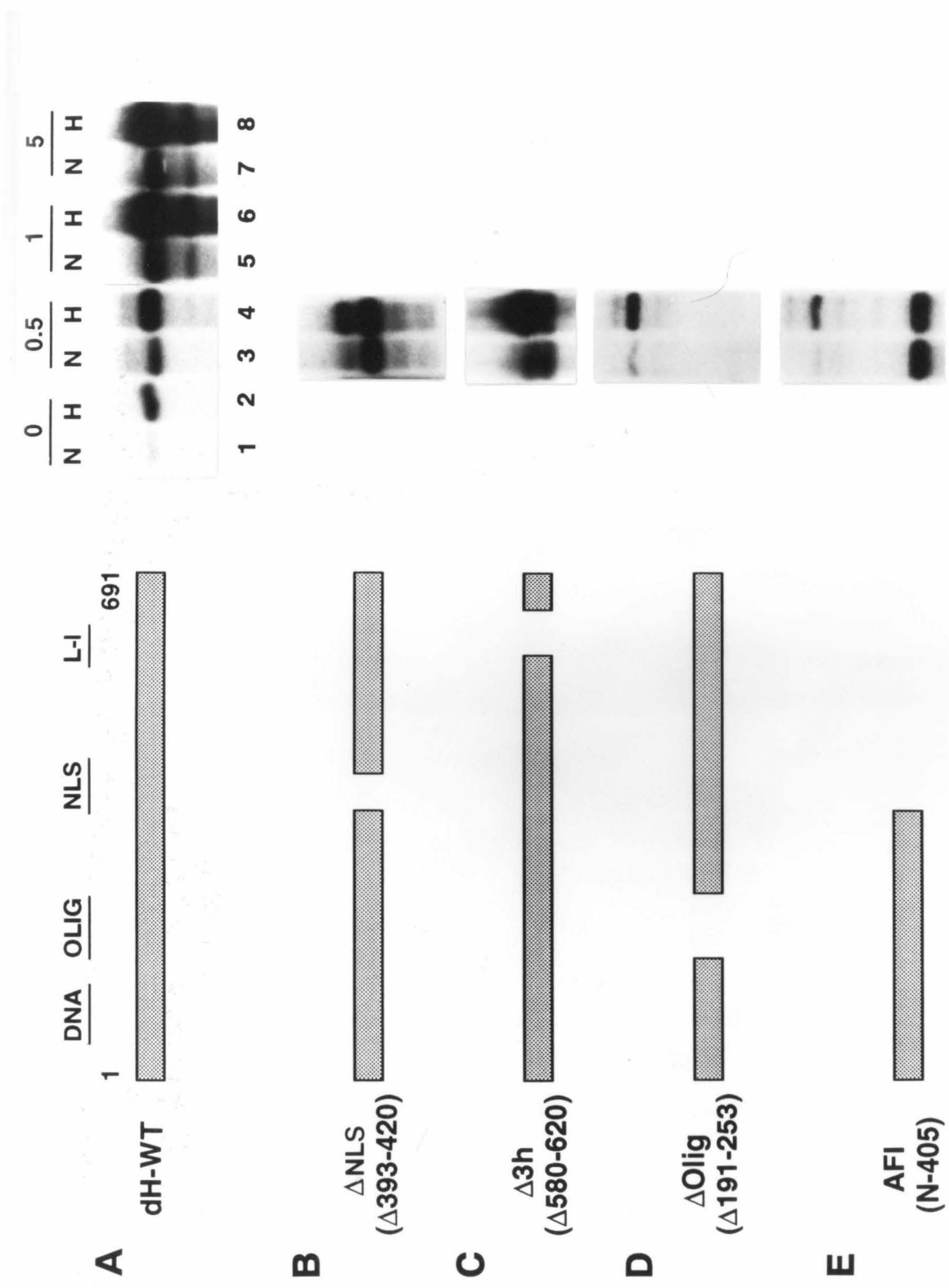
positions of protein standards are indicated at top: thyroglobulin (MW 669,000), ferritin (MW 440,000), human IgG (MW 150,000).

Figure 10. Subcellular distribution of dHSF mutants by indirect immunofluorescence. The mutants and cell lines expressing them used for these assays are the same described in figure legend 8. NS, nonshock; HS, heat shock. For LRR (**A**) cells expressing LRR were induced with 1mM CuSO₄ for 1, 2 and 3 hours as indicated at the top of micrographs in NS panel and for one hour in the HS panel. For K405M (**B**) and L404P (**C**) cells were induced with 1mM CuSO₄ for three hours in the NS and HS panels. For Q403L (**D**) cells were induced for one hour in both panels. Cells were grown at 25°C and after CuSO₄ induction were subjected to heat shock for 30 minutes at 37°C. Cells were fixed, permeabilized and incubated with monoclonal anti-dHSF antibodies. Primary antibody was visualized by fluorescein conjugated goat anti-mouse IgG.

Figure 11. A hypothetical model of interaction of HSF's nuclear localization region with different factors. Single letters indicate amino acids of the NLS region in single letter code. The outline letters indicate amino acids whose mutation affected the NLS function. For a detail description see discussion section of text.

Fig. 1

A**B**



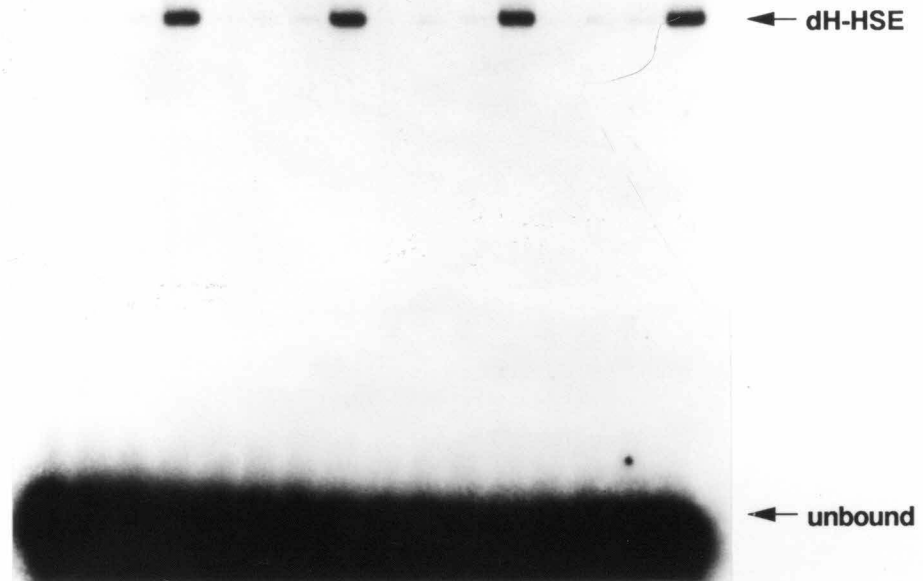
127

Fig. 3

A

induction with
1mM CuSO₄
in hours

0		1		2		3	
C	Nu	C	Nu	C	Nu	C	Nu
n	h	n	h	n	h	n	h



DNA

Western

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

B

NS				HS			
V0	669	440	150	V0	669	440	150
↓	↓	↓	↓	↓	↓	↓	↓

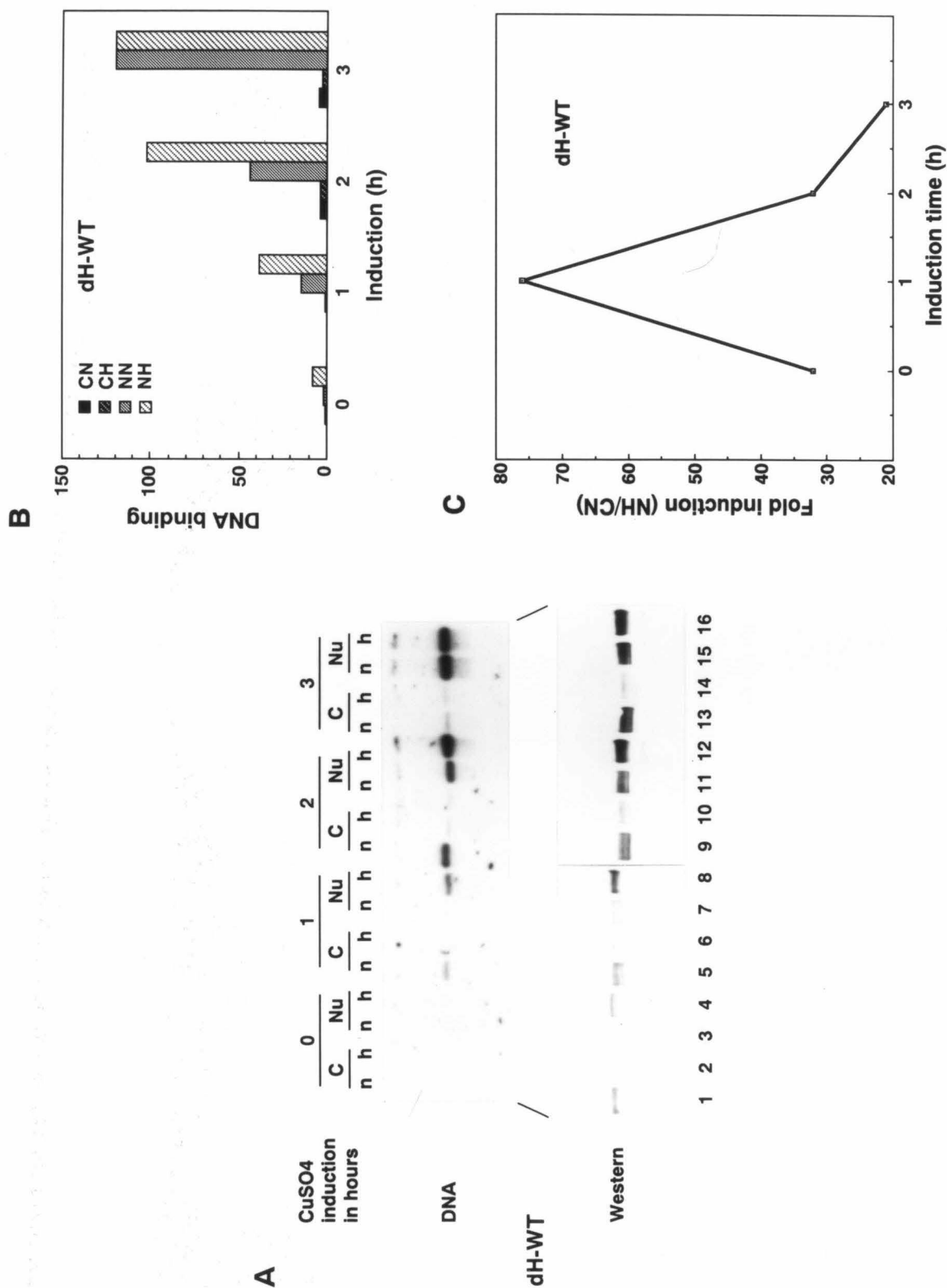
Western

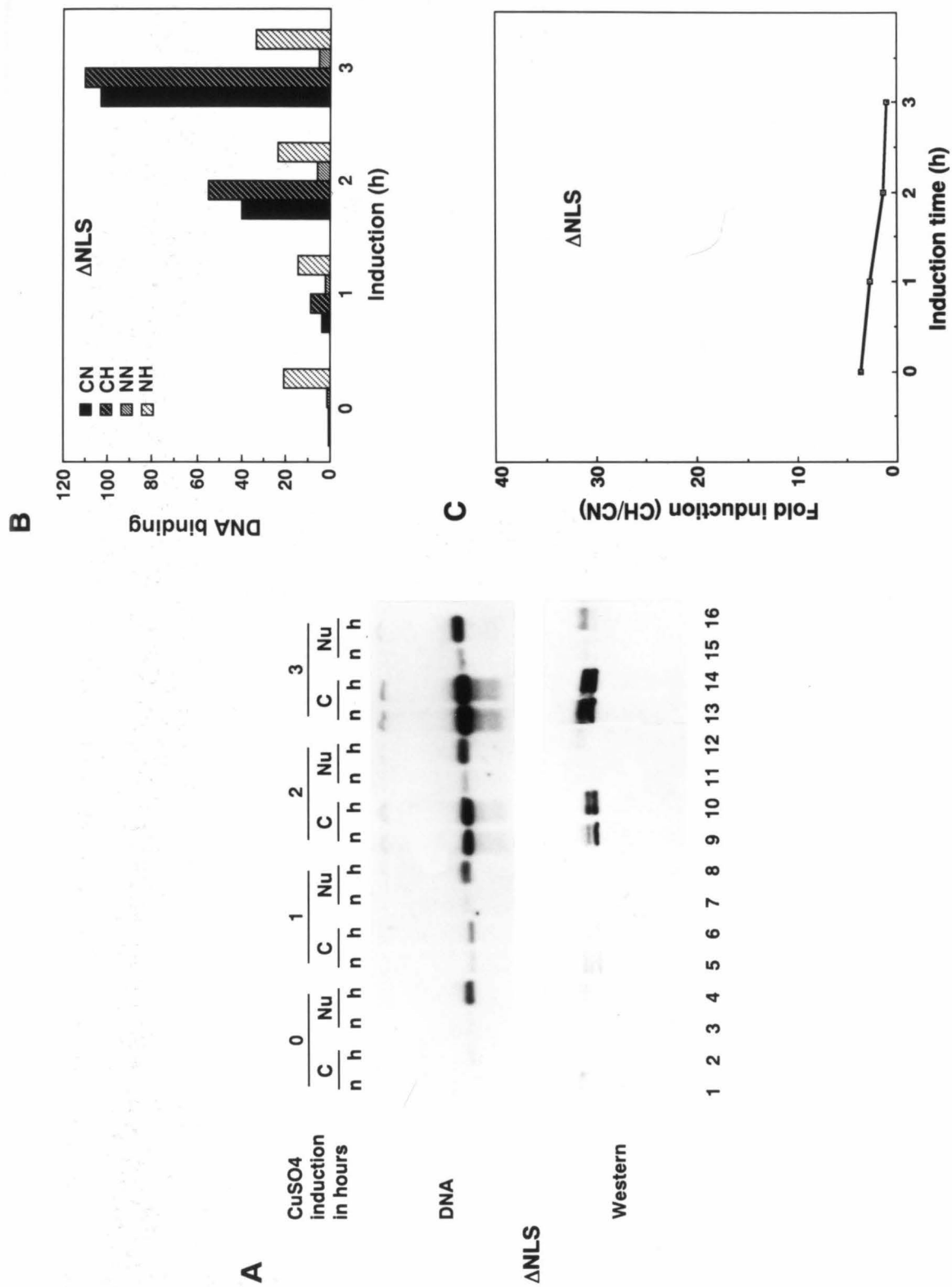
DNA

NS

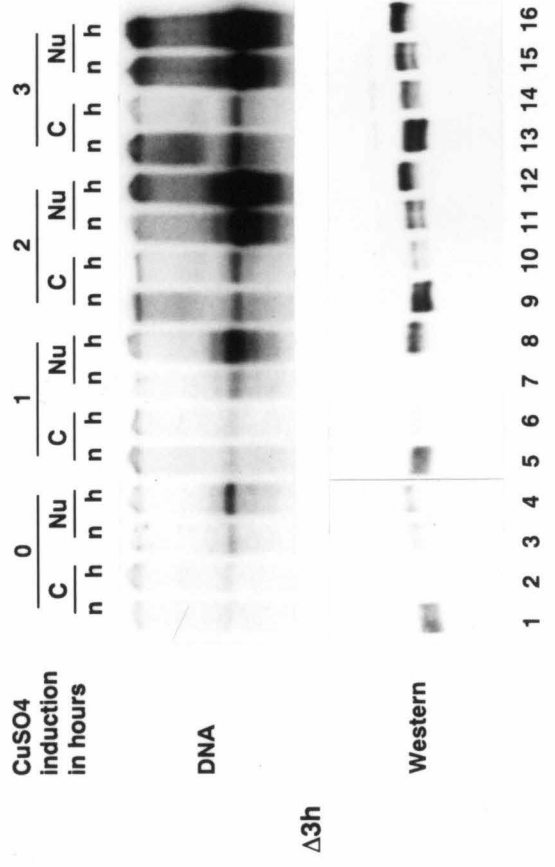
HS

Fig. 4

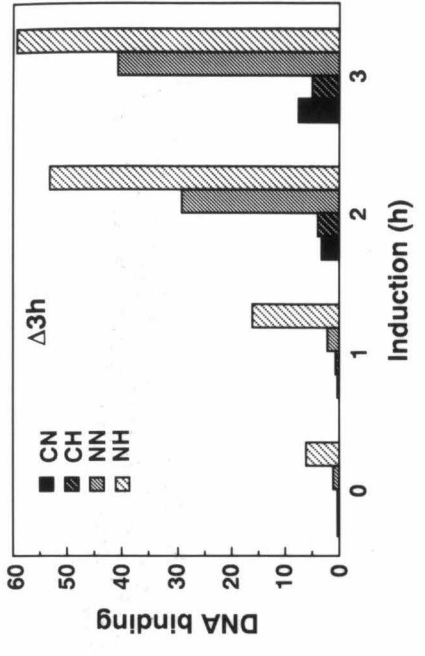




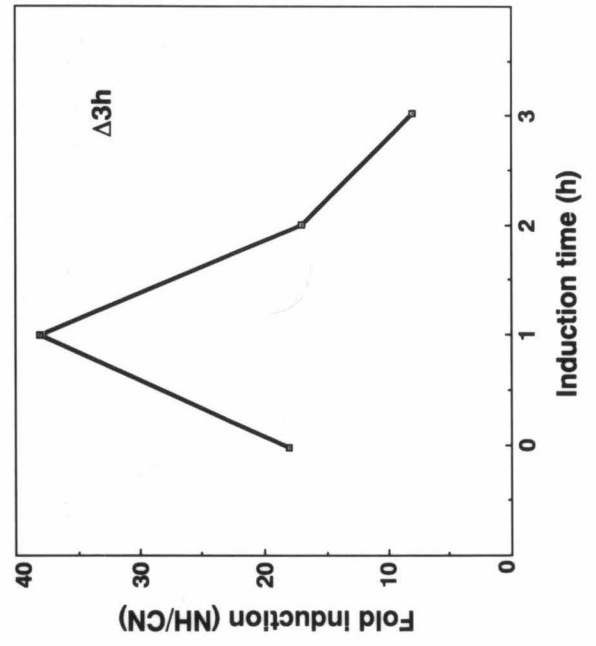
A



B



C



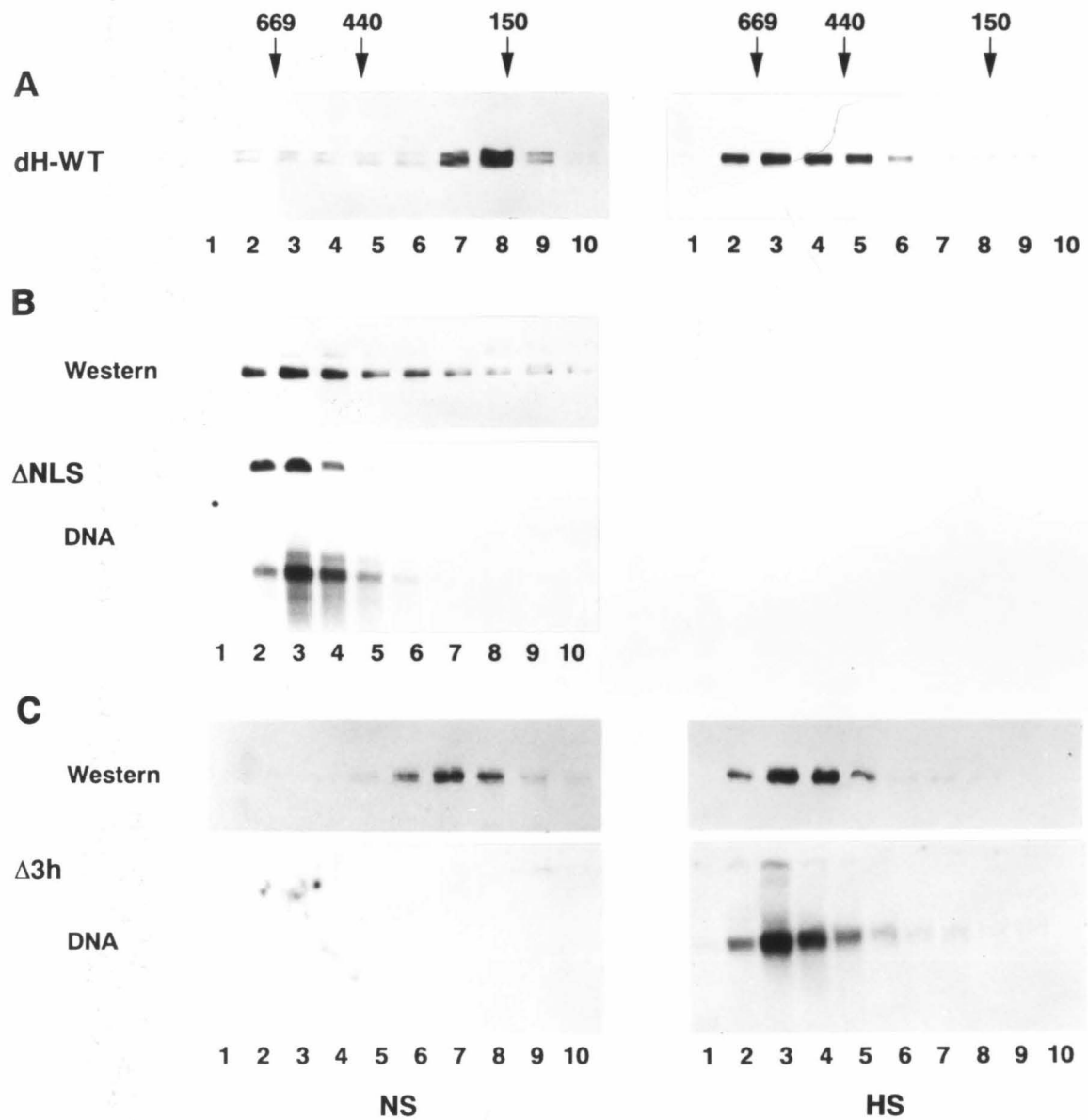
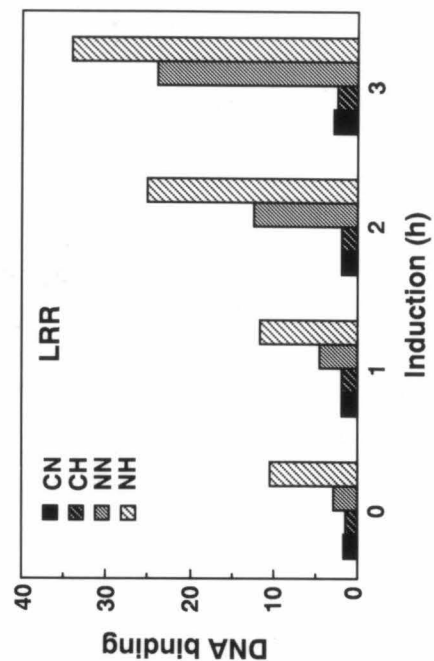


Fig. 8 A



A

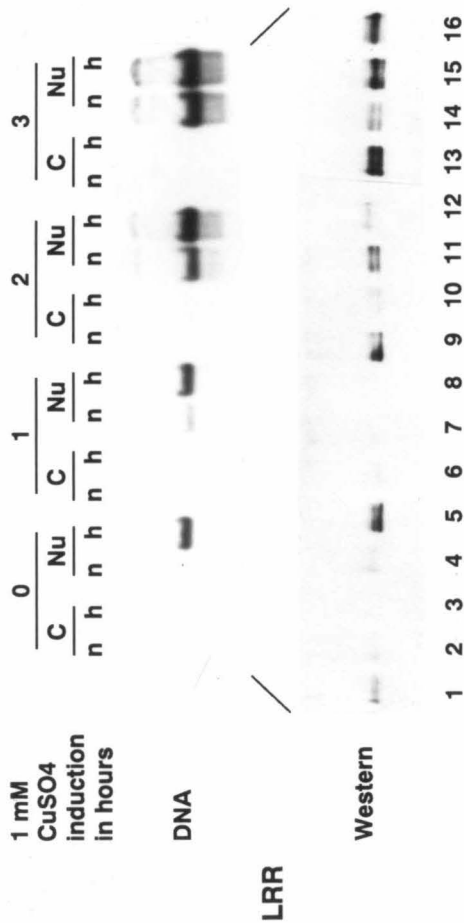
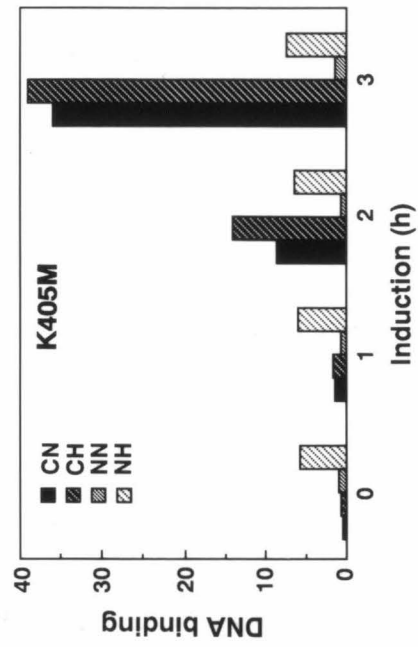
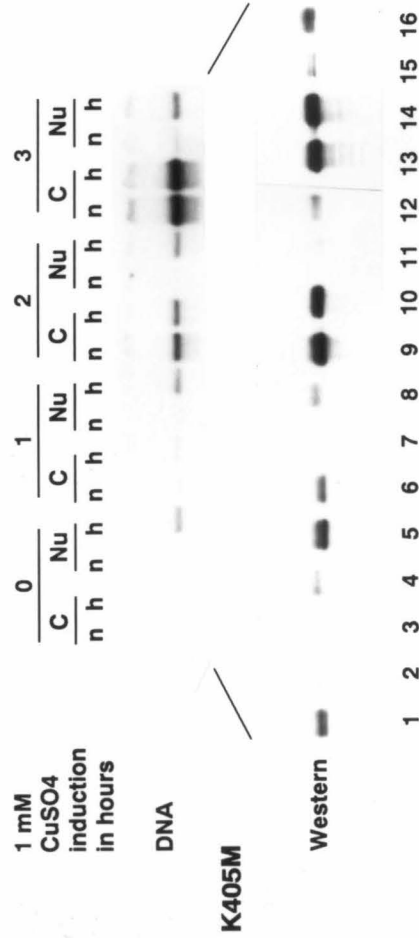
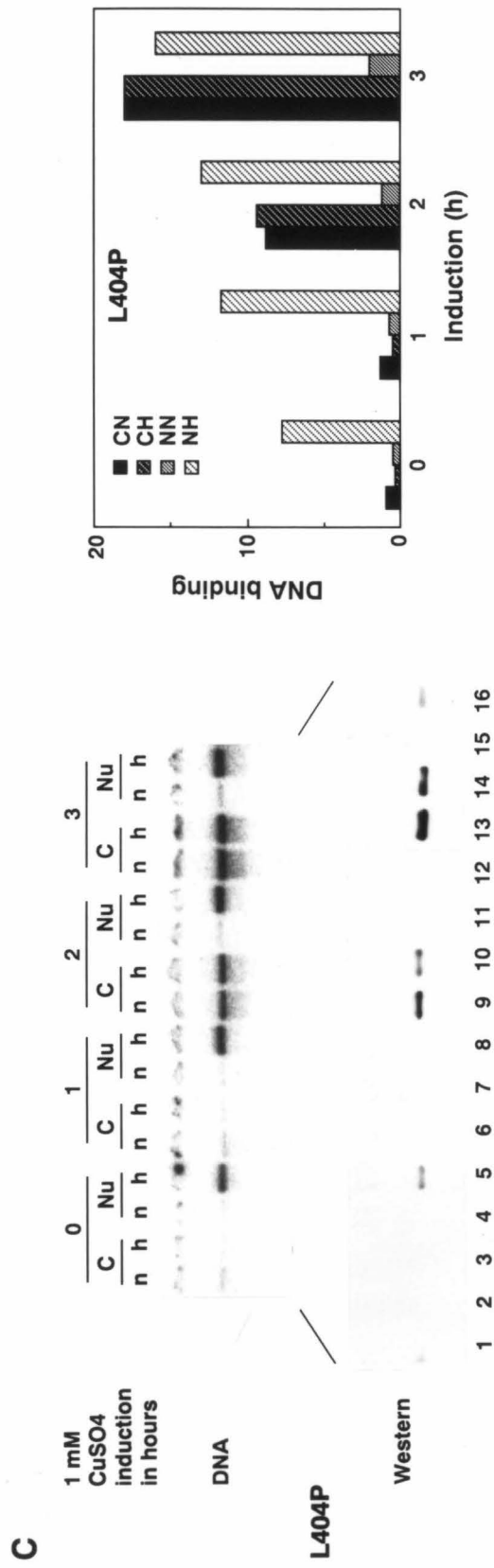


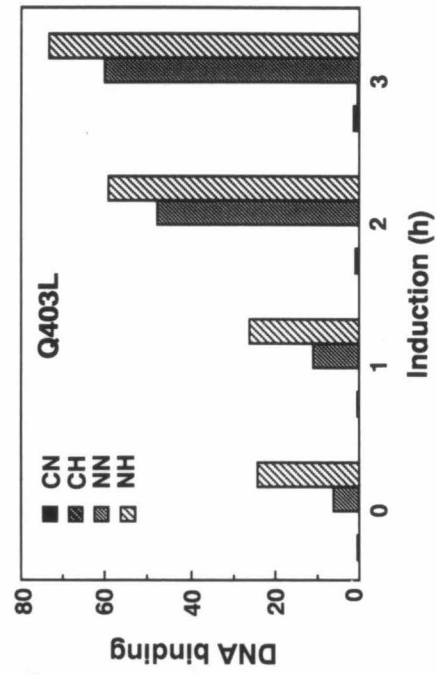
Fig. 8B



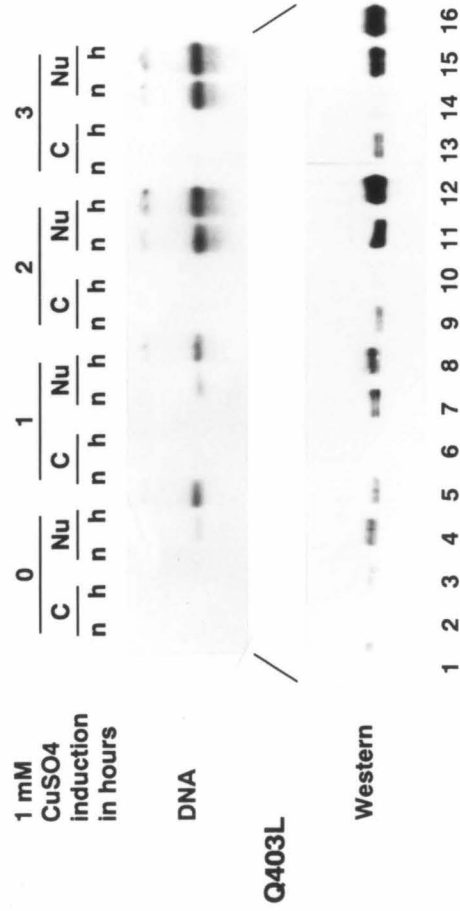
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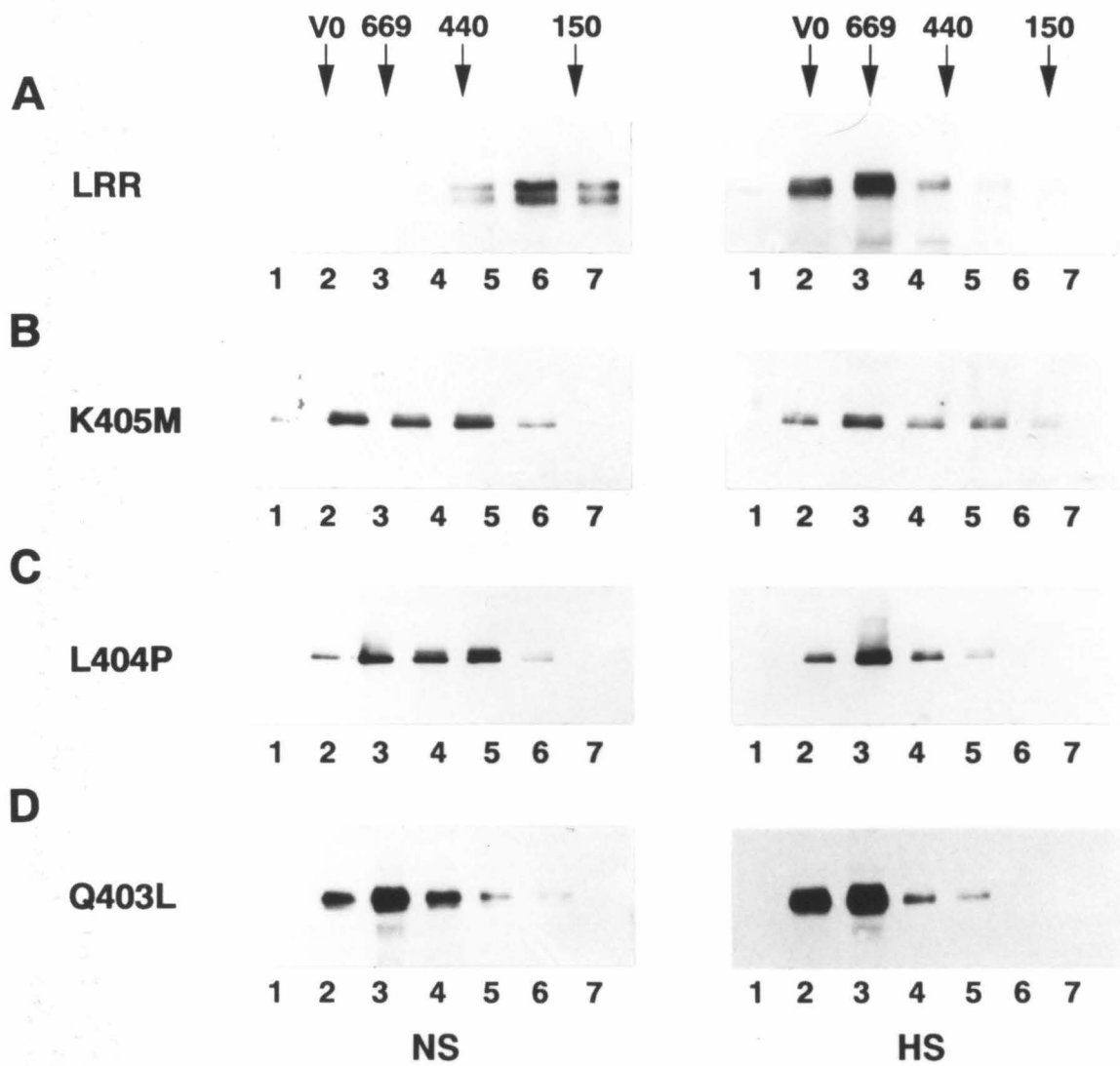


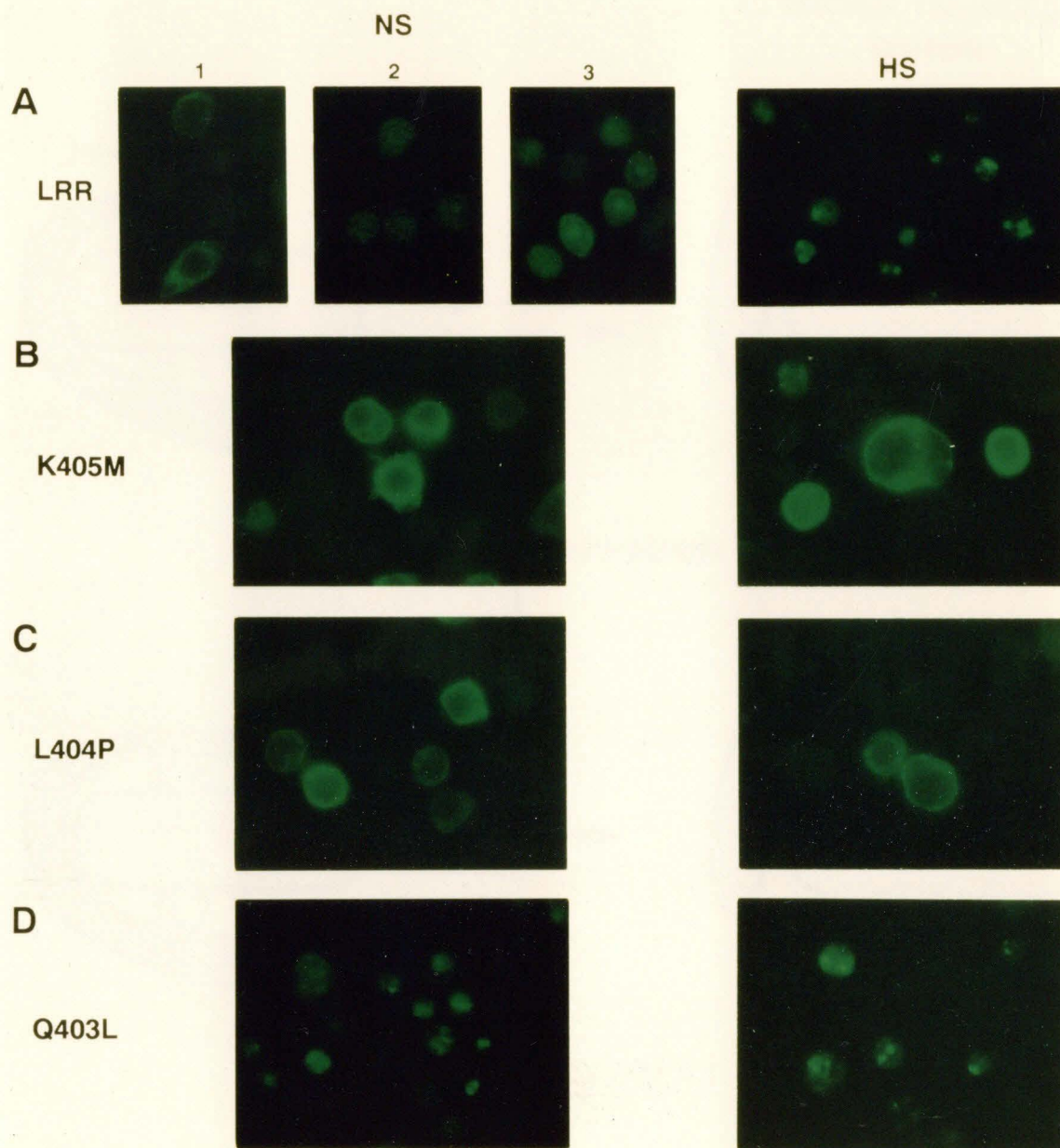


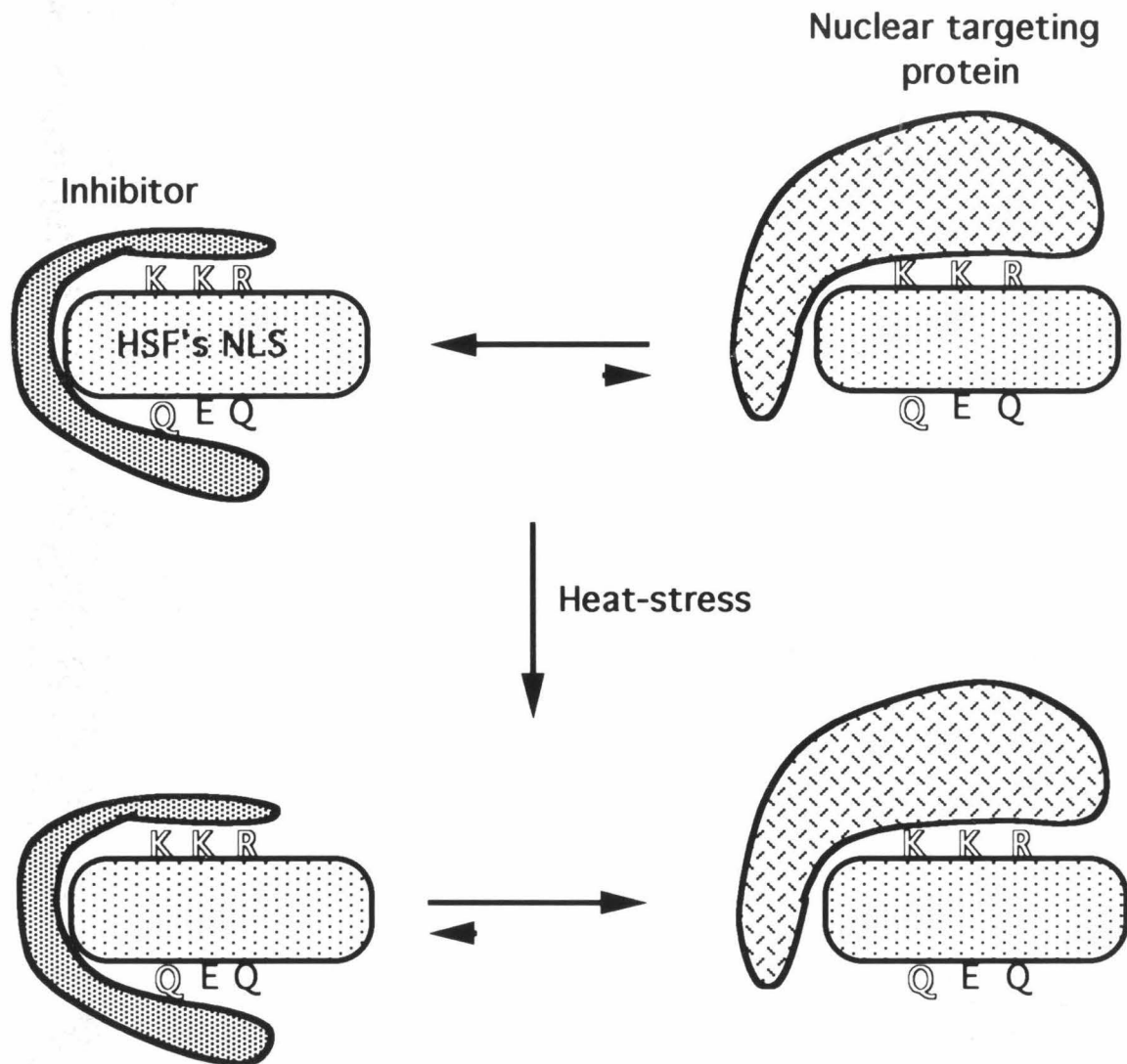


D









Chapter 4

Analysis of the Oligomeric State of the *Drosophila* Heat Shock Factor and the Mechanism Controlling the Monomer to Trimer Transition

Ebrahim Zandi and Carl S. Parker

Abstract

Oligomerization of the *Drosophila* heat shock factor (dHSF) is regulated by heat shock and is required for DNA binding. The state of the oligomerization and the mechanism regulating this step were analyzed. It is shown that the dHSF forms trimers in the solution in absence of DNA as well as when bound to DNA. It is further shown that cytosolic fraction of cultured *Drosophila* cells and embryos contain an activity which inhibited the DNA binding of the nuclear HSF *in vitro*. The inhibition is specific for the HSF and is not due to a degradation of protein. This activity, although it inhibited the DNA binding, was not able to transform trimeric HSF to the monomeric form quantitatively *in vitro*. This and further *in vitro* analysis of the monomer-to-trimer and trimer-to-monomer transitions of the dHSF suggest the existence of a multi-component complex for regulation of the dHSF's activity.

Introduction

The oligomeric nature of eukaryotic transcription factors is relevant to their biological activity and specificity. The determination of the oligomeric state as well as the regulation of the monomer to oligomer transition of specific transcription factors is essential for the understanding of mechanisms controlling their activity. A large number of transcriptional activators bind to their DNA cognate site as homo- or hetero-dimers. Mechanisms controlling specific homo- or hetero-dimer formations contribute largely to the regulatory steps of several groups of transcription factors such as the AP-1 family, steroid hormone receptors, the STAT family, NF- κ B, and cAMP regulated factors (reviewed by Wingender, 1990; Hunter and Karin, 1992; Karin, 1994). Activities of all these factors are regulated by extra cellular signals (Karin, 1994).

Activation of heat shock transcription factor (HSF), in response to heat shock in insect and mammals, occurs at multiple steps including subcellular distribution, oligomerization, DNA binding, and transcriptional activation (see chapter 1). HSF can bind to DNA only in an oligomeric conformation. In *Saccharomyces cerevisiae*, (sc) although the oligomerization is not regulated by heat shock, it has been shown that the scHSF exists as trimeric units in solution in the presence or absence of HSE (Sorger and Nelson, 1989). In the case of the HSF from *Drosophila* (dHSF), a hexameric state was reported based on the mobility of the factor on native gel and cross-linking experiments performed with the recombinant dHSF (Clos *et al.*, 1990).

In this study the state of oligomerization of the native dHSF in the cell extracts from non shocked and heat shocked cells was analyzed by methods of cross-linking, gel filtration, and homo-hetero-oligomer formation. It is shown that, in the presence or absence of HSE, dHSF exists in a monomeric form in the cytosol of the non shocked cells, and in a trimeric state in the nuclear fraction of the heat shocked cells. It is further shown that cytosolic, monomeric dHSF can be activated to the oligomeric form by *in vitro* heating.

The involvement of other cellular factors that may control the monomer to trimer transition and the DNA binding of the dHSF was analyzed as well. Evidence is presented that a yet unknown activity is capable of inhibiting specifically the DNA binding of the dHSF *in vitro*. The mechanism of the inhibition may include a transfer of the trimer to monomer conformation. Attempts for purification of this activity are also discussed.

Results

Drosophila HSF forms trimers in response to heat shock

The inactive form of *Drosophila* HSF is always found in the cytosolic fraction of the non shocked cells. On the other hand, the active, DNA-binding form of the HSF is found in the nuclear fractions of the non shocked and heat shocked cells (Clos *et al.*, 1990; Zandi *et al.*, 1995). The mobility of the HSF present in the cytosolic non shocked and nuclear heat shocked fractions of SL2 cell were compared on native and denaturing polyacrylamide gels.

Drosophila SL2 cells were grown at 25°C and heat shocked at 37°C for 30 minutes prior to preparing extracts. Cytosolic and nuclear fractions were prepared as described in the Materials and Method section. The mobility of the HSF present in these extracts was first analyzed in the presence of a ³²P end-labeled double stranded oligonucleotide containing the heat shock elements (HSEs) from *Drosophila* hsp70 promoter (Parker and Topol, 1984). Duplicates of each sample were run on separate gels. One of the gels was used for autoradiography to visualize the DNA-protein complex, and the other gel was transferred to nitrocellulose and dHSF was visualized by specific anti dHSF antibodies by western blot. Results of these experiments are presented in Figure 1. The HSE-HSF complex can be seen only in the nuclear fraction of heat shocked cells (Figure 1A, lane 3), but not in the cytosolic fractions of non shock or heat shock (Fig. 1A, lanes 1 and 2). As is seen on the native western, dHSF is

recognized by the antibody as a smear midway down in lane 1 of the Figure 1B which corresponds to the cytosolic fraction of the non shocked cells. A weak smear of dHSF is detected in the cytosolic fraction of the heat shocked cells (lane 2, Figure 1B). The dHSF in the nuclear heat shock fraction is detected in the lane 3 as a sharp band in the top part of the Figure 1B. The position of this band corresponds to the position of the HSE-HSF complex on the DNA-binding gel (Figure 1A). In Figure 1B, the relative mobility of bands in the lanes 1 (cytosol non shock) and 3 (nuclear heat shock) to each other shows that HSFs in the cytosol of non shocked cells have a faster mobility than the HSFs in the nuclear fraction of heat shocked cells. In addition, the smear on lane 1 of Figure 1B indicates that the non shock form of HSFs are heterogeneous in their structure. These observations confirm the notion that activation of the dHSF for DNA binding is accompanied by the transition from a lower level of molecular organization to a higher level of oligomerization state as a result of heat shock.

Next, the state of the oligomerization of the dHSF was examined by cross-linking and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Cytosolic non shocked and nuclear heat shocked fractions of SL2 cells were incubated with 2mM EGS, and cross-linked proteins were separated on a 5% SDS-PAGE. The dHSF was visualized by western blotting. Non-cross-linked HSF migrates at the molecular weight range of about 97 kDa (Figure 1C) which is consistent with its monomeric form. Cross-linked HSFs from non shocked cells migrate at two positions on the gel: one is a strong band at the 97 kDa range and the other is a

weak band at the 200 kDa range. The ratio of the 97 kDa to the 200 kDa migrating HSF bands was estimated to be about 20:1. The 200 kDa band represents dimeric HSFs. The presence of dimers of the HSF in the cytosol of non shocked cells may be because some cells were partially stressed during the extract preparation, or that HSF was partially activated by the cross-linking condition. The cross-linked HSF from the nuclear heat shock migrates on the gel at a position corresponding to about 300 kDa range (Figure 1C). This molecular weight range is consistent with the trimeric form of the dHSF. From these analyses it is clear that the dHSF forms trimers as a result of heat shock.

To examine the oligomeric state of the HSF when bound to HSE, UV cross-linking experiments were performed. A specific HSE oligo was prepared (see Materials and Method) that is capable of covalent cross-linking to HSF when irradiated by ultraviolet light. This DNA probe was end-labeled with ^{32}P and mixed with either cytosolic non shocked or nuclear heat shocked fractions of SL2 cells under the same condition that was used for the gel shift assay shown in Figure 1A. Under this condition, the HSE-HSF complex is specific and HSEs do not bind to other cellular proteins. After 15 minutes of binding, reaction mixtures were UV irradiated and protein-DNA complexes were separated by SDS-PAGE and visualized by autoradioagrophy. To confirm that UV irradiation did not result in the non specific HSE-protein complexes, one half of the UV cross-linked reaction mixtures were compared to a set of similar non cross-linked reaction mixtures on a native gel. The comparison did not show any non-specific HSE-

protein complexes (data not shown). A picture of the autoradiogram of the SDS-PAGE is shown in Figure 1D. In the case of the cytosolic non shocked fraction, there is a doublet at the MW range of 97 kDa in addition to two weak smears at 200 kDa and 300 kDa ranges. The 97 kDa bands correspond to the monomeric dHSF that are cross-linked to HSEs. For the heat shock extract, bands at similar positions are seen; however, all bands in the heat shock lane are 15 to 20 times stronger than the corresponding bands in the non shock lane (Figure 1 D). This suggests that, as expected, dHSF in the non shocked cytosol do not form a stable complex with HSEs. Nonetheless, the sensitivity of this assay (due to cross-linking and ^{32}P) allows the detection of very weak interactions between the HSE and HSF seen in the cytosol fraction. The weak bands at higher MW range in the cytosol of the non shocked fraction represent HSFs from partially stressed cells, or HSFs that are activated by UV *in vitro*.

The lower molecular weight bands in both cases are stronger than those of higher molecular weight ones. This may be due in part to the screening of the cross-linking sites on the DNA from UV by the trimeric conformation of the HSFs on HSE. The presence of the dimers and trimers on the gel, however, indicates that in some cases three or two HSFs are cross-linked to one HSE. It is also possible that cross-linking among proteins occurs as well, although less efficient, which indicates that there is sufficient close protein-protein contacts when HSFs are bound to DNA.

The trimer formation of HSF on HSE was further examined by analysis of hetero oligomer formation between the full length and truncated HSFs. This method was previously used to show that ScHSF forms trimers on HSE (Sorger and Nelson, 1989). In this method, the number and the size of the homo- and hetero-oligomers formed between the full length and truncated protein give information about the oligomeric state. For a protein that forms trimers, four complexes are expected by this method (see Figure 1E). The largest and smallest complexes consist of homo-trimers of the full length and the truncated protein, respectively. The two other complexes consist of two full length and one truncated or two truncated and one full length protein (see Figure 1E). Because all of these complexes differ significantly in their size and thus their mobility on the native gel, they can be distinguished in a gel shift assay. Figure 1E shows such an experiment where the endogenous dHSF forms hetero-oligomers with truncated HSFs that were expressed in the SL2 cells. One of the truncations has an internal deletion from amino acid 460 to 536 (this mutant is designated as Δ Bst). The other HSF truncation used for this assay lacks 172 amino acids from its carboxy terminus (N-519, Figure 1E, lanes 5 and 6). Extracts of cells that were transfected with vector-only shows only one band which is specific for HSF-HSE complex in the heat shocked cells. Both Δ Bst and N-519 when expressed in the SL2 cells form hetero-trimers with endogenous HSF as is seen in Figure 1E. There are four bands clearly visible in each case of these factors, although intensities among these bands in each lane are different. The homo-trimers are stronger

than the hetero-trimers. This may be due to a more stable conformation of homo trimers when bound to HSEs. Nonetheless, the fact that four bands are clearly visible shows that dHSF forms trimers.

In conclusion, all above experiments show that the dHSF forms trimers in the presence or absence of HSE. The trimer formation is seen mainly in the heat shock cell extracts indicating that heat shock induces the trimerization of the dHSF.

Trimerization of the dHSF can be induced by *in vitro* heating of the cell extracts.

It was examined whether *in vitro* heating of the cytosolic extract of non shocked cells would induce oligomerization of the HSF. For this experiment a gel filtration assay was used to compare monomers and oligomers of the dHSF. The gel filtration column was calibrated by determining the elution volumes of thyroglobulin (669 kDa), apoferritin (440 kDa), human IgG (150 kDa), BSA (66 kDa) and cyanocobalamin (29 kDa). The presence of the HSF in the eluted fractions was assayed by western blot using monoclonal anti-dHSF antibodies. First, extracts of the cytosolic non shocked and nuclear heat shocked SL2 cells were fractionated on the column (see Materials and Method). Figure 2A shows the elution profile of the cytosolic non shocked dHSF. The majority of dHSF elutes at the positions that are consistent with the monomeric form (150 kDa). The elution profile of nuclear shows that HSF mainly elutes at the 669-440 kDa

range that is consistent with its oligomeric state. To examine the effect of *in vitro* heat shock, the cytosolic fraction of non shocked cells was incubated at 37°C for 15 minutes prior to fractionation on the gel filtration column. The elution profile of this experiment is depicted in the Figure 2C which shows that a significant amount of the dHSF elutes at the high molecular weight (669-440 kDa) range. Thus the oligomerization of the dHSF can be induced *in vitro*.

It was also examined whether *in vitro* activated HSF would bind to HSE. A quantitative binding of the *in vitro* activated dHSF to HSEs was not observed, however (data not shown). This might indicate that the mere oligomerization is not sufficient for the activation of DNA binding and some yet unknown modifications of the HSF are required for full DNA binding activity. This idea is supported by the observation that recombinant HSF has much lower affinity for HSE compared to the HSF when isolated from the nuclear heat shock cells (data not shown).

***In vitro* reconstitution of the non-DNA-binding state of the dHSF**

Results from chapter 2 and 3 suggested the existence of a regulatory factor(s) which may maintain the dHSF in its inactive conformation in the cytosol of the non shocked cells. In addition, in cells in which protein synthesis has been inhibited, the DNA-binding of the HSF is activated upon heat shock and deactivated after returning the cells to their normal condition; a second heat shock re-activated the HSF in these cells (reviewed by Voellmy, 1994). This suggests a

mechanism for the de-activation and re-activation of the DNA-binding of the HSF *in vivo*. Here, it was attempted to reconstitute the inactive state of the dHSF *in vitro*. In these experiments, the active state is considered as the DNA binding form of the factor. The source of the active form of the dHSF was the nuclear extract of the heat shocked Kc cells. Gel shift was used as the assay system. Cytosolic extracts from *Drosophila* cell lines (Kc and SL2) as well as whole *Drosophila* embryos were used as a source for the regulatory activity. The assay was based on incubating the nuclear extract of the heat shock cells with the cytosolic extracts. Several variables of the incubation conditions were examined. It was found that when the nuclear extract of the heat shocked cells were incubated with either of cytosolic extracts of non shocked SL2, Kc cells, or whole embryos at room temperature (23-25°C), but not on ice, the DNA binding activity of the nuclear HSF was decreased as a function of the increasing amount of the cytosolic extract (Figure 3A). The decrease of DNA binding was not due to the degradation of the HSF as it was examined by western blot (Figure 3B). The specificity of the inhibition of the dHSF DNA binding by these extracts was examined by using other DNA-binding proteins. As it is shown in Figure 3C, the activity of the human octamer binding protein 1 (Oct1, He *et al.*, 1989 and references therein) was not affected in the same reaction in which the DNA binding of the dHSF is inhibited. Thus, this inhibitory effect is specific for the dHSF. This activity was heat (60°C) and proteinase K sensitive (data not shown).

To examine whether this activity would transform the dHSF from the trimeric state to the monomeric form, the nuclear extract of the heat shocked cells was incubated either with the cytosolic extract of non shocked *Drosophila* embryos or with assay buffer at room temperature for 45 minutes followed by the fractionation of each reaction mixture on the gel filtration column. The elution profiles of the dHSFs in these fractions are shown in the Figure 4A and 4B. Interestingly, the embryo extracts prepared for these experiments contain a very low amount of the HSF as was examined by gel filtration and western blot (data not shown). Therefore, the HSF seen in the gel filtration analysis that follows is from the nuclear extract. As is seen in Figure 4A, when nuclear extract was incubated with buffer, the majority of HSFs elute 669-440 molecular weight range which is consistent with the oligomeric form. Incubation of the nuclear extract with the embryo extract results in some of the HSFs eluting in the lower molecular weight range, which indicates that some of the HSFs are in the monomeric form. In this experiment, the transition of HSF from the oligomeric state to the lower molecular weight conformation is not quantitative. At this point, the reason for this is not clear, and from this experiment it can not be concluded that the mechanism for the de-activation of the DNA binding of the HSF by embryo extract include a trimer to monomer transition. Further analysis of this activity using recombinant HSF (rHSF) instead of the nuclear extracts in a similar set of experiments showed that the oligomeric state of the rHSF was not

affected quantitatively by this activity, although the DNA binding of the rHSF was affected to some extent (data not shown).

It was attempted to purify the component(s) of the activity by methods such as ion exchange, affinity, and gel filtration. The activity could not be recovered when ion exchange columns or the rHSF affinity column were used. The only purification step in which about 50% of the activity was recovered was the fractionation of the crude cytosolic embryo extract by gel filtration (data not shown). According to the gel filtration analysis the activity is found in the 27-40 kDa eluates. This partially purified activity was not able to efficiently transform trimeric dHSF to the monomeric form (data not shown). This activity, which reduces the affinity of the dHSF for HSE, may be a part of a larger complex that is required for the complete de-activation process of HSF.

Is dHSF associated with an inhibitor in the monomeric conformation?

The comparison of the mobility of the non-cross-linked and cross-linked HSF from cytosolic non shocked cells showed no significant difference in the molecular weight. This indicates that the dHSF may not be associated with an inhibitory factor which could maintain the HSF in the monomeric state. It is still possible that the regulatory factor would be a small molecule and addition of it to the HSF would not contribute to the mobility of the HSF on the SDS-gel and therefore not be detectable. To examine whether such molecule exists and can be isolated from HSF, a large quantity of the monomeric dHSF from the cytosolic

fraction of non shocked cells was isolated by gel filtration. This partially purified HSF was heated *in vitro* to induce the oligomeric state under similar conditions as above, following the fractionation on the gel filtration column. Over 90% of the HSF eluted in the 669-440 molecular weight range (data not shown). Those fractions that did not contain HSF were analyzed for their ability to inhibit DNA binding of the HSF either separately or when pooled together; these separate and pooled fractions were also tested for their ability to transform the trimer HSF to monomer. Neither of the single fractions nor the pooled fraction affected either the DNA-binding or the oligomeric state of the HSF (data not shown). These experiments, although not conclusive, can be interpreted in different ways. The first possibility is that the HSF in the monomeric conformation is not associated with an inhibitory factor. On the other hand, if there is an inhibitor, it is possible that either *in vitro* heating have inactivated the putative inhibitor, or some component that is necessary for the re-association of the inhibitor with the HSF is missing. In addition, the possibility that the partially purified 27-40 kDa activity, which inhibited the DNA binding (see above), might be the missing component was examined. The combination of the 27-40 kDa activity with the above pooled fractions did not restore the monomeric conformation of the HSF (data not shown).

To examine whether the crude cytosolic extract contains all of the components necessary for the trimer to monomer conversion *in vitro*, HSF in the crude extract of the cytosolic non shocked cells were activated to oligomerize *in*

vitro at 37°C followed by incubation at 25°C for various periods of time. It was thought that incubation at 25°C would mimic an *in vivo* recovery condition. The analysis of the oligomeric state of the HSF showed that majority of HSFs were oligomeric after *in vitro* heat shock and remain oligomeric also after being incubated at 25°C indicating that oligomerization was not reversed (data not shown). This observation can be interpreted as either the cytosol does not contain all the necessary components for the trimer to monomer conversion, or *in vitro* heat shock has de-activated some components of the activity needed for the conversion.

Taken together, it seems that the oligomeric state of the HSF is regulated by a multi-component complex which is very sensitive to assay conditions. To develop purification strategies to isolate factors that regulate the oligomeric state of the dHSF, further detailed characterization of the 27-40 kDa inhibitory activity seems necessary, as well as finding a condition for *in vitro* trimer to monomer transformation.

Discussion

The oligomerization state of the *Drosophila* heat shock factor free in solution or bound to a HSE with three half-sites is examined here using two separate methods. First, chemical and UV cross-linking analysis showed the dHSF forms trimers in the absence of, or bound to, HSE in response to heat shock. Secondly, when truncated HSFs are co-synthesized with the endogenous full length factor in the cell cultures, four oligomeric complexes, two homo-oligomeric and two hetero-oligomeric, are observed. Since three HSFs bind to one HSE containing three half-sites, it seems that each HSF molecule interacts with one half-site. If this is in fact true, an interesting question is how the three-fold symmetry of the HSF trimer is imposed on the two fold symmetry of the HSE. A partial answer may lie in the linker region which connects the DNA-binding and the oligomerization domains. The flexibility of the linker region may confer sufficient conformational flexibility to the trimer allowing the binding to HSE with dyed symmetry. Ultimately the X-ray analysis of the HSF-HSE complex will answer this question.

Most of the DNA-binding proteins, especially transcription factors, bind to their DNA cognate sites as dimeric units (Steitz, 1990). Dimerization is necessary for the binding and function of transcription factors (Kouzarids and Ziff, 1989; Hai and Curran, 1991). Usually, binding of transcription factors as a dimer or a multimer of dimers is cooperative. Cooperativity increases the binding efficiency and specificity (Hai and Curran, 1991). For the HSF it has been shown that

binding to HSEs containing one or two half-sites is very weak and in most cases not detectable, indicating that the cooperativity of at least three HSFs on three half-sites is necessary for the efficient binding (Xiao *et al.*, 1991).

Why do HSFs bind as trimers and what is the advantage of a trimeric unit compared to a dimeric one in regards to the function and regulation of the factor? HSF is a very potent transcriptional activator compared to other transcription factors. Does this somehow relate to the trimeric binding of the HSF to the promoter? One advantage of trimeric binding versus dimeric or monomeric binding would be the higher concentration of the HSF at the site of transcription. It is known that when the concentration of an activator at the promoter sites is increased, e.g., by inserting more binding sites to the promoter; the rate of the mRNA synthesis increases accordingly. Another advantage is that monomer to trimer transition confers novel regulatory mechanisms to the HSF. It is a fact that in insect and mammals the trimerization of the HSF is regulated by heat shock. HSF is kept in a monomeric form incapable of DNA binding; heat shock induces the trimerization and DNA binding. The mechanisms of the trimerization and, equally important, the trimer to monomer conversion are not clear. Data presented in this report suggest that a yet unknown activity is able to inhibit the DNA binding activity of the HSF, perhaps by a transformation of the trimer to monomer. The maintenance of the monomeric conformation of the HSF seems to be an important issue for the regulatory mechanisms involved. So far it is not clear whether HSF is maintained in the monomeric conformation solely by

an intrinsic property of its own or if the assistance of a regulatory factor is needed. The fact that the molecular weight of the cross-linked cytosolic HSF did not show an upshift in the SDS-gel indicates that the maintenance of the monomeric state may be an intrinsic property of the HSF, although the resolution of the SDS-PAGE may not allow for the detection of the association of a small factor. On the other hand, there are observations that support the involvement of an associated factor to the HSF: i) recombinant HSF is oligomeric at all times; ii) the DNA-binding activity of the nuclear HSF is inhibited by a partially purified activity from the cytosol, and the mechanism may involve in part the trimer to monomer transition; iii) the cytosolic residency and the monomeric state of the HSF when expressed in the cell cultures depends on its expression level and is titratable. These observations taken together indicate that HSF needs other cellular components to maintain the monomeric conformation. However, thus far it has not been possible to isolate the activity which would transform the recombinant HSF from the oligomeric state to the monomeric conformation. In addition, interacting regions of the HSF with the identified inhibitory activity remains unknown.

Attempts to purify a single component as an inhibitor were not successful here. A reason for this could be that there might be a multi-component complex required for the process of trimer to monomer transition of the HSF. When the partially purified monomeric fraction of the HSF was activated *in vitro* and fractionated on the gel filtration column, an activity that would inhibit the DNA-

binding or establish the monomer form of the HSF was not found in any of single or pooled fractions suggesting that either there was no factor associated with the HSF or, if there were such factor present, it could not re-associate with HSF by itself. The re-association of an inhibitor to HSF might need the assistance of some other cellular factors. On the other hand, the activity that inhibited the DNA binding also did not transform trimeric HSF to the monomeric form quantitatively. It may be that, in the preparations of the inhibitory activity, some factors needed for establishment of the monomer conformation were present in very low concentrations or were not functional *in vitro*. Since the inhibition of the DNA binding was efficient compared to the establishment of the monomeric form, it is reasonable to argue that the inhibitory activity that inhibits the DNA binding is different from the factor which would establish the monomeric conformation. Therefore, there is a reasonable possibility for the existence of a multi-component complex that regulates the tertiary conformational changes of the HSF. Such a complex would de-activate HSF in cells during the recovery from a heat shock period. If such a complex exists, a hypothetical mechanism for its activity would include the de-activation of the DNA-binding state of the active-HSF by covalent modifications (e.g., phosphorylation) followed by reconstitution of the monomeric conformation. The establishment of the monomeric form would include a refolding of the trimeric HSFs and, if there is a factor needed for the maintenance of the monomeric state, the catalysis of association of such factor to HSF. This hypothetical mechanism suggests that part

of the function of such complex would be the catalysis of the folding and refolding of the HSF. This function not only would be needed during the recovery from a stress period but also during the translation of new HSFs for establishing the monomeric conformation of the factor. This hypothetical scenario further suggests that such complex would consist of enzymes and structural components. The definition of the presumptive complex matches that of the molecular chaperones (Hartl and Martin, 1992). Several heat shock proteins such as hsp70, hsp60, hsp90 and small hsps are shown to be integral parts of molecular chaperones (Hartl and Martin, 1992; Hendric and Hartl, 1993). Since hsp70 and hsp90 are thought to play a role in the regulation of the HSF in human cells (for a review see Voellmy, 1994) and they are also found to be associated with and regulate the function of several other transcription factors such as steroid hormone receptors (Bohen and Yamamoto, 1994), they, in addition to other hsps, might be integral components of the hypothetical complex.

In conclusion, data presented in this report with conjunction of data from chapters 2 and 3 suggest that the process of the monomer to trimer or trimer to monomer transition of the HSF is a complex event and it may require the participation of several cellular factors organized in a multi-component complex. To understand and to be able to reconstitute these processes *in vitro*, it is necessary to isolate and identify the components of this complex.

Materials and methods

DNA constructs that were used for SL2 transfection, the maintenance of cell cultures, transfection's condition, and preparation of mini cell extracts are described in chapters 2 and 3.

The native gel electromobility shift, SDS-PAGE, and western blotting procedures are described in chapter 3.

The following protease inhibitors were added to buffers for all cell extract preparations and inhibition assays: 1.5 mM phenylmethyl-sulfonyl fluoride, 150 μ g of leupeptin per ml, 25 μ g of pepstatin A per ml, 25 μ g of benzamidine-HCl per ml, 5.0 chymostatin per ml, 10 μ g of aprotinin per ml, 300 μ g of 1,10-o-phenantroline per ml, and 50 μ g of bestatin per ml.

Preparation of cytosolic and nuclear extracts

Nuclear extract was prepared essentially as described by Parker and Topol (1984). Fifteen liter mid-log *Drosophila* Kc cells (Echalier and Ohanessian, 1969) were grown in the spinner flasks at 25°C and heat shocked at 37°C for 20 minutes in a water bath as described in Parker and Topol (1984). For preparing cytosolic and nuclear extracts, non-shocked or heat shocked cells were harvested by centrifugation at 5000 rpm for 5 minutes in the Beckman J6 centrifuge. The cell pellet was washed by resuspending in 10 times its volume of cold buffer A: 15 mM KCl, 10 mM Hepes pH 7.9, 2 mM MgCl₂, 0.1 mM EDTA. The Washed cells

were resuspended in 50 ml of the cold buffer A supplemented with 1 mM DTT and incubated on ice for 10 minutes. NP-40 was added to a final concentration of 0.5% and cells were broken by homogenization in a glass dounce homogenizer. The cell homogenate were centrifuged at 10,000 rpm for 15 minutes in a JA-21 Beckman rotor. The supernatant was transferred in a new tube and centrifuged at 20,000 rpm for 30 minutes. The supernatant is the cytosolic fraction that was used for experiments. The nuclear pellet from the first centrifugation was resuspended in 5 ml per liter of the original cell culture in a buffer composed of 9 parts buffer A and 1 part buffer B (1 M KCl, 50 mM Hepes pH 7,6, 30 mM MgCl₂, 0.1 mM EDTA, and 1 mM DTT). The crude nuclear suspension was transferred to Beckman 45Ti polycarbonate centrifuge tubes, and 4M ammonium sulfate (pH 7.9) was added to a final concentration of 0.36 M. The nuclei were lysed by gentle rocking on a Lab-quake mixing device at 4°C for 30 minutes. The lysate was centrifuged at 35,000 rpm for 60 minutes in a 45Ti Beckman rotor. The clear supernatant was collected and proteins were precipitated by addition of 0.25 g ammonium sulfate per ml of supernatant. The precipitated proteins were collected by centrifugation at 35,000 rpm for 15 minutes. The pellet was resuspended in buffer D (10% glycerol, 25 mM Hepes (pH 7,6), 100 mM KCl, 0.1 mM EDTA, 0.01% Triton X100, and 1mM DTT) and subsequently dialyzed against buffer D for a minimum of four hours. The extract was centrifuged at 10,000 rpm for 10 minutes and frozen in small aliquots at -70°C. The protein concentration at this point was usually between 10-15 mg/ml.

Conditions for chemical and UV cross-linking

Proteins were cross-linked in 20 μ l reaction volume in 150 mM NaCl and 50 mM phosphate buffer pH 7.0 by adding 2 μ l of 20-40 mM ethylene glycol *bis* (succinimidylsuccinate), EGS, (Pierce). EGS was dissolved in DMSO. The cross-linking were performed at the room temperature (23-25°C) for 15 minutes. After cross-linking one volume of SDS-loading buffer was added to the reaction mixtures and heated at 95°C prior to separation of proteins on a 5% SDS-PAGE (Laemmli, 1970). HSF was visualized by western blot using monoclonal anti HSF antibodies.

UV cross-linking was carried out in gel shift assay reactions with intense 254 nm ultraviolet light source (Fotodyne Incorporated, Model # 3-3000). Cross-linking was performed on reactions incubated on ice. The UV source was placed ~1-2 cm above the reactions (in Eppendorf tubes) for 15 minutes. One volume of SDS-loading buffer was added and reaction mixture was heated at 95°C loaded on a 5% SDS-PAGE. Protein-DNA complexes were visualized by autoradioagraphy of dried gel.

Preparation of HSE oligo for cross-linking

The 5-[-N-(p-azidobenzoyl)-3 aminoallyl]-deoxyuridine triphosphate (N3dUTP) was synthesized and purified as described in Bartholomew *et al.*, (1990) with the exception that the reaction was carried out at room temperature over night instead of the three hours suggested in their protocol.

For cross-linking of HSF to HSE, the N3-dUTP incorporated HSE was synthesized. All reactions were performed at reduced light. Single stranded oligonucleotides (5' - GACGATGCGGCCGCGCCTCGAATGTTCGCGAATTCGCGGCCGCGAACTAG-3' and the complementary strand of this oligo) that contain three half-sites (underlined sequences) of the first heat shock element in the *Drosophila* hsp70 promoter (Parker and Topol, 1984) were used as templates to synthesized double stranded HSE by Klenow enzyme. Each single stranded oligo was annealed to a complementary 14-mer primer at its 3'-end by heating at 100°C for 5 minutes and cooling down to room temperature over a period of 30 minutes. Annealed oligoes were used in a reaction mixture that contained: 300 μ M of dGTP, dATP, and N3dUTP each, 100 μ M dCTP, 20 μ M α -³²P-dCTP (30,000-60,000 Ci/mmol), 10 mM MgCl₂, 20 mM Tris pH 7.6, 1 mM DTT, and two units of Klenow enzyme. Reactions were incubated at 37°C for 30 minutes. The reaction mixtures of both oligos were mixed and heated at 100°C for 5 minute at reduced light and cooled down to the room temperature over a period of 30 minutes. Labeled double stranded oligos were purified on a native polyacrylamide gel. For each gel shift assay, a mixture of 20,000 cpm of the labeled double stranded oligo was used.

Gel filtration chromatography

For analytical gel filtration, 500 μ l of cytosolic extract or 50 μ l of nuclear extract was loaded on a superdex 200 HR 10/30 (0.5x30 cm, Pharmacia) column

that was equilibrated in the buffer NPO [150 mM NaCl and 50 mM Na₂HPO₄ (pH 7,0)]. The column was run at 0.5 ml per minute flow rate. The void volume of this column is about 6 ml. Oligomeric HSF eluted between 6.5 and 8 ml, and monomeric HSF eluted between 8.5 and 10 ml. Fractions of 0.5 ml or 1 ml were collected. Proteins in each fraction were separated on SDS-PAGE and HSF was visualized by western blotting (see chapter 3).

For preparative gel filtration, the preparative superdex gel (Pharmacia) was packed in a Xk 26 Pharmacia column (3 x 100 cm) in the buffer NPO. The loading volume for this column was 5-10 ml. This column was run at 1 ml per minute flow rate, and fractions of 10 ml were collected. All gel filtrations were performed at the cold room on a Pharmacia FPLC GP-250 instrument.

Assay condition for the inhibition of the HSF's DNA binding

In a typical 20 μ l inhibition reaction 0.1 μ l nuclear extract of heat shocked cells and 1 to 5 μ l of the cytosolic extract of the non shocked cells or embryos were incubated in buffer NPO that was supplemented with 1 mM DTT, 1 mg/ml BSA, (protease inhibitors) at 25°C for 15-30 minutes. The gel shift cocktail containing ³²P labeled HSE was added, and reaction mixture was incubated at room temperature for additional 15 minutes prior to loading on a 4% native polyacrylamide gel in 0.5 x TAE buffer. Gel was developed and exposed to the film or phosphor imager screen (Molecular dynamics) as described in chapter 3.

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Figure legends

Figure 1. Analysis of the oligomeric state of the *Drosophila* HSF. (A) and (B), Mobility of the HSF-HSE complex on the native gels. Cytosolic non shock (CN), cytosolic heat shock (CH), and nuclear heat shock (NH) extracts of Kc cells were incubated with a ³²P labeled HSE and analyzed on native gels. (A) shows a picture of an autoradiogram and (B) shows a duplicate of the (A) that was probed with anti HSF antibodies; (C), Chemical cross-linking of HSF. Nuclear heat shock (HS) and cytosol non shock (NS) were cross-linked by 2 mM EGS and separated on a 5% SDS-PAGE. HSF was visualized by specific monoclonal antibodies; (D), UV cross-linking of HSF to HSE. The picture shows an autoradiographic analysis of the HSF-HSE complex; (E), Truncated/full length HSF complexes separated on native gel. SL2 cells were transiently transfected with Paco vector, PacoΔBst, or PacoN-519. Non shocked (n) and heat shocked (h) whole cell extracts were prepared for each transfection. The extracts were incubated with ³²P labeled HSE and analyzed on a 4.5% native gel. 3L, three full length; 2L+1S, two full length and one truncated; 3S, three truncated.

Figure 2. Oligomerization of the dHSF by *in vitro* heat shock. Oligomerization state of the dHSF in the cytosolic fraction of the non shocked SL2 cells (A), the nuclear heat shock (B), and cytosol non shocked fraction that was incubated at 37°C for 10 minutes prior to fractionation (C) were analyzed by gel filtration on a superdex 200 HR 10/30 column. Fractions of 1 ml were collected and analyzed by western blot. The elution profiles are shown. The

approximate elution positions of protein standards are indicated: thyroglobulin (MW 669,000), ferritin (MW 440,000), human IgG (MW 150,000), and BSA (66,000).

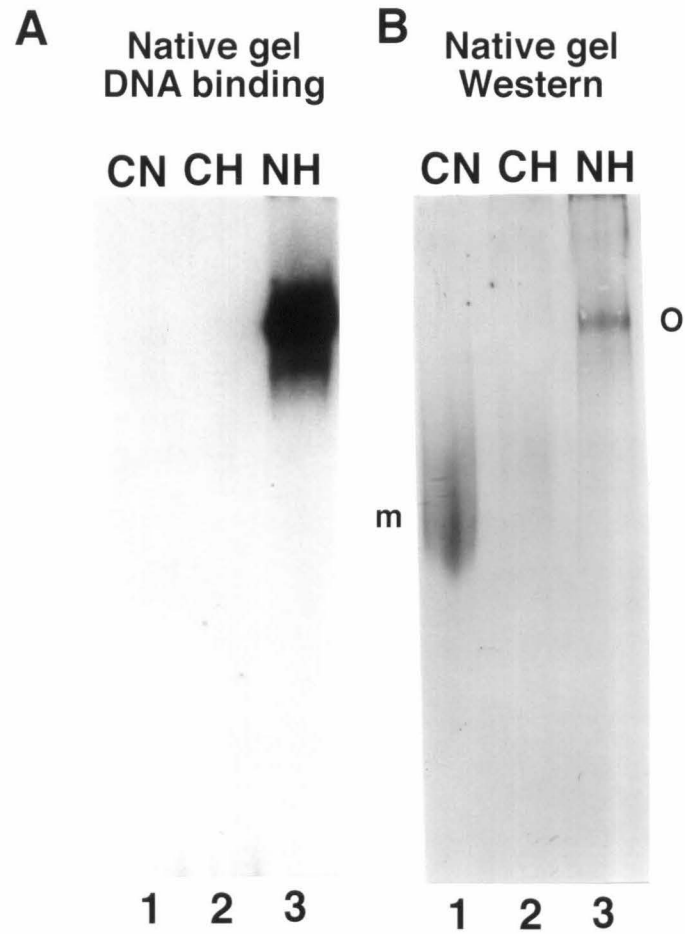
Figure 3. Inhibition of the DNA-binding of the dHSF *in vitro* by cytosolic fraction. Nuclear extract of heat shocked Kc cells was incubated with buffer (0), 1, 3, or 5 μ l of the cytosolic fraction of non shocked SL2 cells for 30 minutes at room temperature prior to the gel shift reaction (see Materials and method). (A), gel shift analysis when gel shift reaction was performed with HSE oligo. (B), one-half of the samples used in the gel shift in (A) were analyzed on SDS-PAGE and western using anti HSF antibodies. (C), The reaction condition is the same as in (A) except 0.1 μ l of nuclear extract of human HeLa cells was included in the reaction as source for the human octamer binding factor 1 (hOCT1). The gel shift reaction was performed with a mixture of HSE and octamer binding site : 5'-GATCCTTTAATAATTTGCATACCCTCA-3' and its complement (Staudt *et al.*, 1988) that were labeled at the 5' end with [32 P]-ATP and T4 polynucleotide kinase.

Figure 4. Effect of the cytosolic non shocked fraction of *Drosophila* embryos on the oligomerization state of the HSF in the nuclear heat shock fraction. Superdex 10/30 HR fractions profiles of the nuclear extract (50 μ l) from the heat shocked Kc cells (A) and the same amount of the nuclear extract which was incubated with 450 μ l of a *Drosophila* cytosolic embryo extract for 45 minutes at room temperature prior to fractionation (B). The gel filtration conditions as well

as western analysis are the same as described in the legend 2 of the Figure 2 except here 0.5 ml fractions were collected instead of 1 ml.

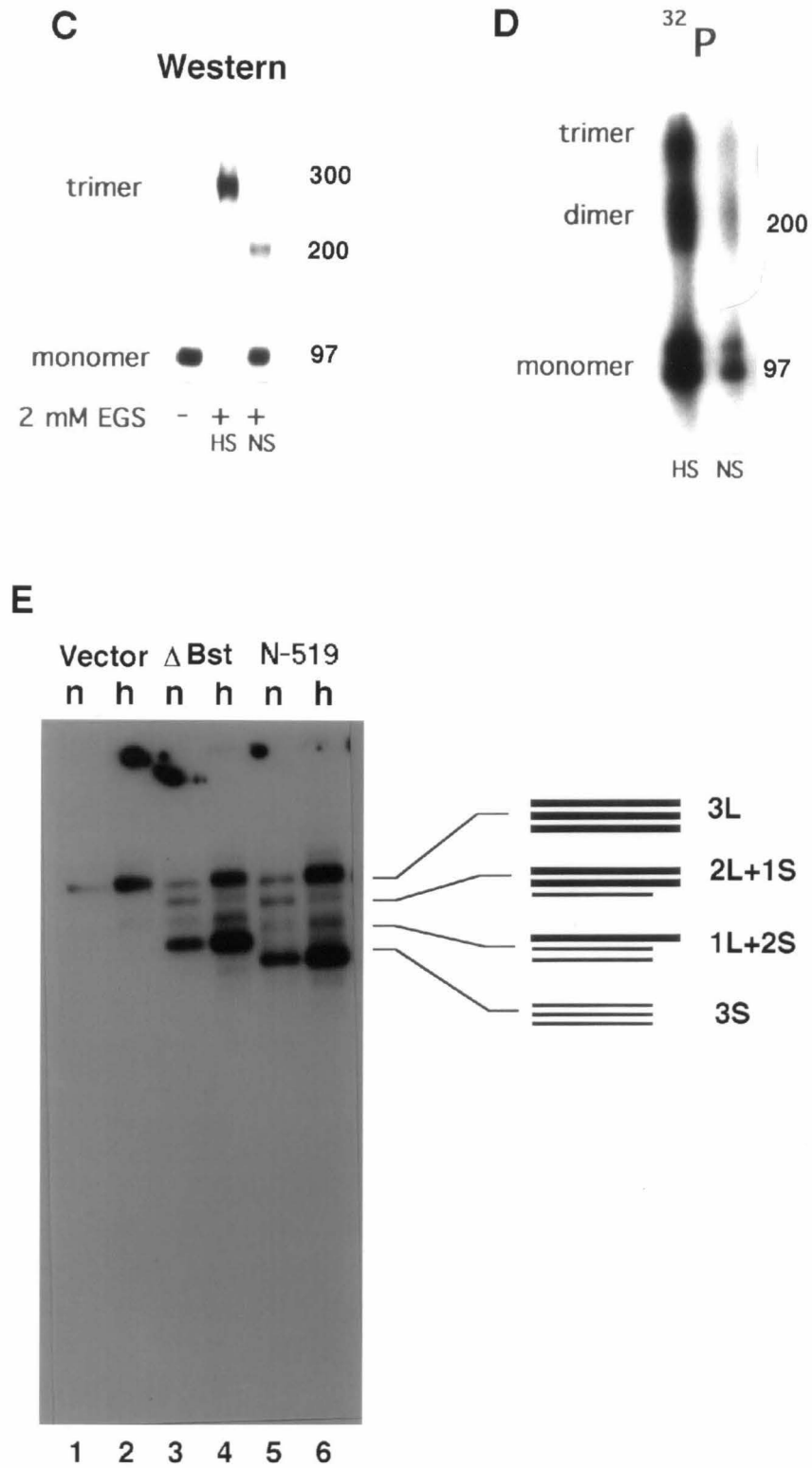
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Fig. 1 A-B



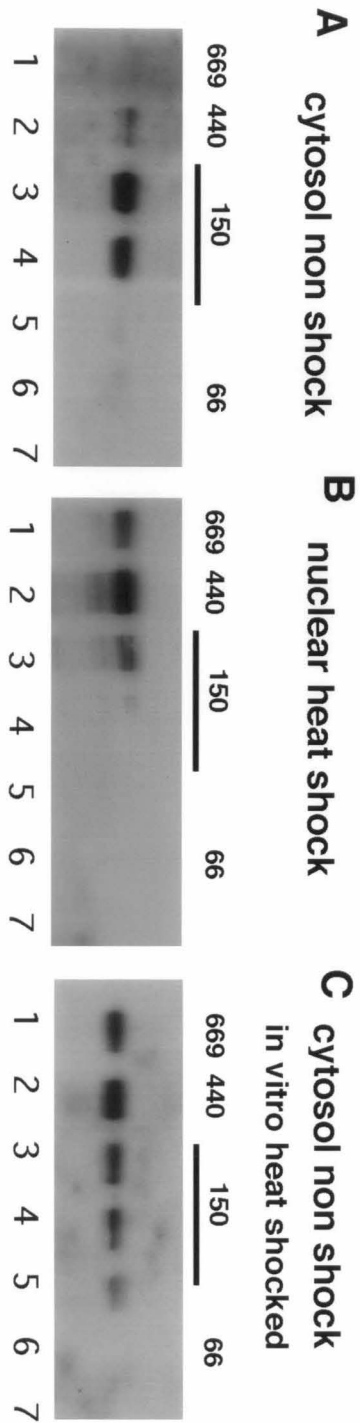
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Fig. 1C-E



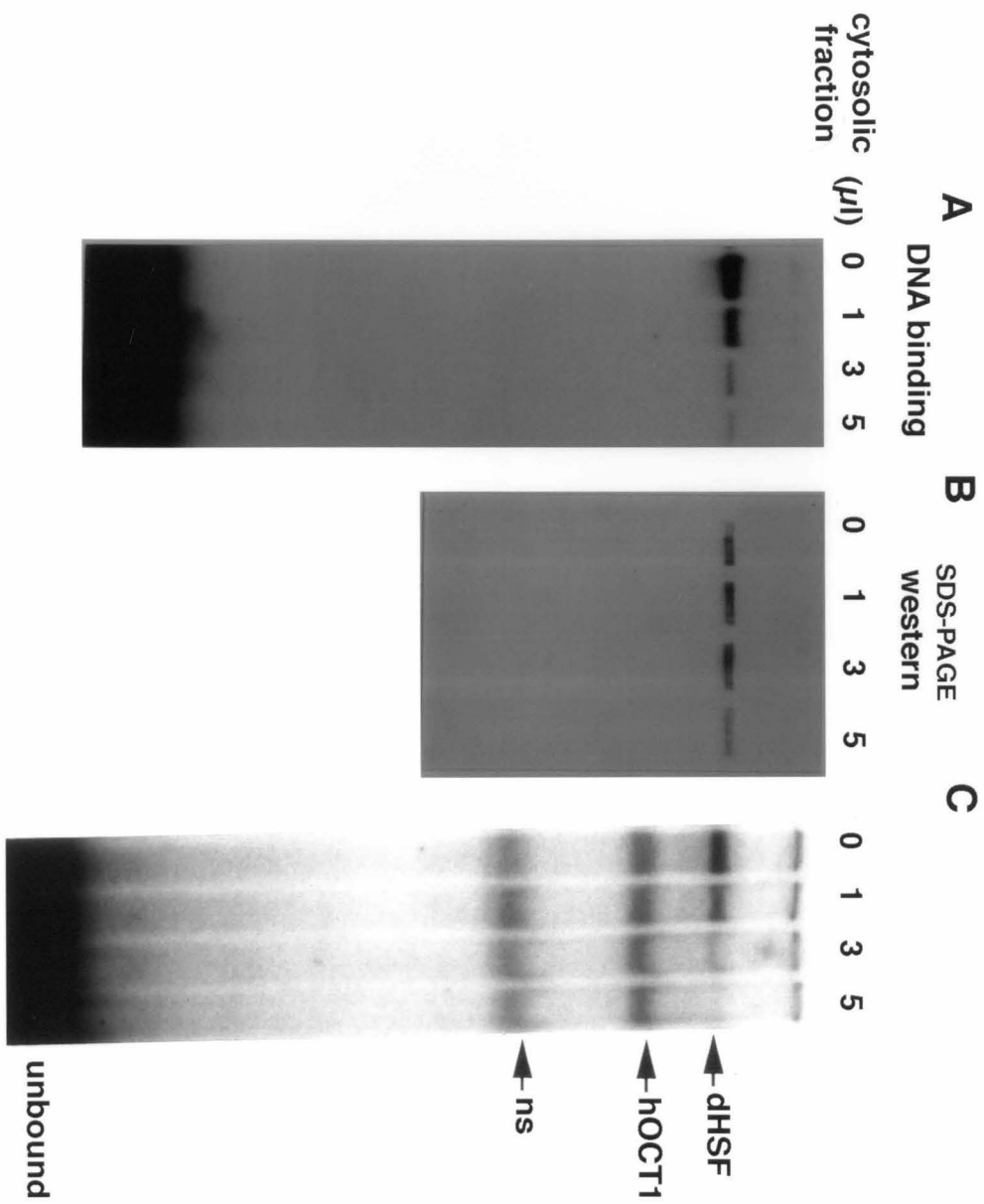
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Fig. 2



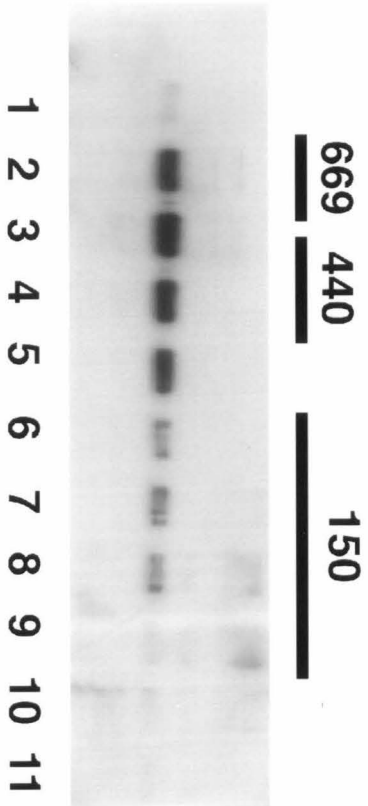
174

Fig. 3



A

nuclear heat shock



B

nuclear heat shock
+
cytosolic fraction

